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TESTING OF COMPOUNDS FOR EFFICACY AGAINST SCHISTOSOMIASIS

ANNUAL REPORT

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A new topical antipenetrant (TAP) was tested in animal test model systems for prevention of penetration of skin by schistosome infective larva (cercariae). The formulation provided complete protection of monkey skin when applied at -1 and -3 days before exposure to *Schistosoma mansoni* cercariae. When the formulation was applied at -7 days before exposure of monkey skin to *S. mansoni*, two of four monkeys were completely protected. The formulation was completely protective against invasion of hamster skin by *S. mansoni* and *S. haematobium* at -1 day but not at -3 or -7 days before exposure to cercariae.

The formulation was not protective against skin invasion by *S. japonicum* cercariae.

When the new formulation was applied simultaneously to skin with a anti-arthopod repellant, no interaction between the two compounds was observed.

A compound (BL 44970) found previously to be highly active as an oral prophylactic drug in the rodent test system was evaluated in *Cebus apella* monkeys and found to be inactive against *S. mansoni* infective larva.

A total of nine candidate compounds were tested for oral prophylactic activity in the mouse test model system. Low levels of protection were obtained which varied from animal to animal. No complete protection was obtained. These compounds are not recommended for further evaluation as prophylactic candidate drugs.

The marmoset monkey was found to be unsatisfactory as a model to study schistosome cercarial penetration dynamics.

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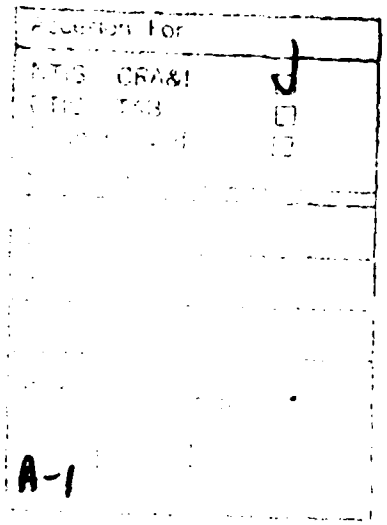
FOREWARD

1. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

2. In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised, 1978).



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INTRODUCTION

The parasitic disease schistosomiasis is endemic in 74 countries in Africa, Asia, the Middle East, South America, and islands of the Caribbean [1]. There are approximately 200-300 million infected persons with nearly 600 million constantly at risk in endemic areas [1]. The disease has shown remarkable and substantial increases due in part to the creation of new water resources such as dams, lakes, and irrigation schemes needed to meet increased energy and food demands. In addition, the introduction of the new water resources has caused dramatic ecological changes to occur in many of the endemic areas for schistosomiasis. Another recently occurring problem in the fight against schistosomiasis has been the emergence of drug-resistant strains in the parasite in Brazil [2,3], and more recently in Kenya [4]. To further complicate the drug-resistant picture, strains of *S. mansoni* which have been maintained for long periods in the laboratory without having any contact with drugs have been found to be resistant to one or more schistosomicides. Two strains used in this program have been found to be resistant. One strain from Kenya has been found to be resistant to niridazole (used as the reference drug in this program) and the other, a strain from Puerto Rico, was found to be resistant to oxamniquine, a drug which is included in the arsenal for use by the Army against *S. mansoni* infections. This emerging problem may compromise the success which has been made against the disease by use of chemotherapy.

An example of the spread of schistosomiasis into an area heretofore free of the disease is the country of Jordan. Up until 1984, Jordan was one of only a few countries in the Middle East free from both susceptible snails and the parasite. But during the past ten years, the threat of the disease becoming established has been growing due to the presence of the vector-snail intermediate host which has defied attempts by the Ministry of Health to prevent its spread by use of chemicals. Indigenous cases have now been discovered nine years after finding the snail intermediate host and eight years after the influx of foreign-infected migrant workers [5,6] thus indicating active transmission.

From the military prospectus, there are three stages of the schistosomiasis disease process in which medical casualties could be expected to occur: (1) skin penetration which is associated with penetration of the infective larva (cercariae); (2) Katayama fever, associated with the initial stages of egg deposition during the chronic phase of the disease; and (3) chronic schistosomiasis, associated with granuloma formation in liver or urinary bladder after the third to fourth month of

infection. Currently, there are no infective larva (cercariae) repellents, and neither prophylactic or suppressive drugs nor vaccines are available for use by the military of the United States. Even if it were possible to recognize early stages of the schistosome parasite in exposed personnel, there are no drugs available to treat early infections. Three drugs are currently available to treat mature infections in humans. These are praziquantel, active against all species of schistosomes infective to man; oxamniquine, active against one species of schistosome infective to man, namely *S. mansoni* (most effective against new world *S. mansoni*); and metrifonate, active against urinary schistosomiasis, namely *S. haematobium* [7]. Strains of *S. mansoni* resistant to oxamniquine [3,4] have been isolated from patients in Brazil and Kenya. It also appears that strains of *S. haematobium* resistant to metrifonate have emerged [8]. This leaves only one antischistosomal agent, praziquantel, with minimal side effects for use in treatment of schistosomiasis at present. Studies to determine if praziquantel is capable of causing drug-resistant schistosomes to occur are in progress.

The lack of specific preventative measures for use by military personnel poses a significant potential problem for military operations which may occur in areas of strategic interest to the United States. Casualties have occurred during previous operations to British, French, Canadian, and American forces.

During the past several decades, many compounds have been tested in an effort to find a formulation which would afford protection against invasion of the skin by *Schistosoma sp.* infective larva (cercariae). This subject has been amply reviewed by several authors [9-13].

Evaluation [14,15] of chemical preparations for their topical prophylactic antischistosomal activity is an ongoing program of the United States Army Medical Research and Development Command whose overall goal is to develop a substance which will protect personnel unable to avoid water contact when operating in areas where schistosomiasis is endemic.

The objective of the program as supported at the Center for Tropical Diseases, University of Lowell is to conduct secondary and definitive test evaluations of compounds showing superior antipenetration prophylactic efficacy against *S. mansoni*, *S. japonicum*, and *S. haematobium* in rodents and/or primates. In addition, these evaluations may be made against other schistosome species and/or drug-resistant forms if requested. Curative and/or suppressive evaluation of compounds can also be carried out when requested.

During this funding period, several protocols were initiated and completed. These protocols are as follows:

1. WRHN-II b. Ten compounds (nine experimental and one reference drug, namely niridazole) were evaluated in mice.

2. WRHN-II c. This topical antipenetrant (TAP) rodent study evaluated the efficacy of the new topical antipenetrant formulation against *S. mansoni* and *S. haematobium* in hamsters, and *S. japonicum* in mice.

3. WRHN-III Ma. This topical antipenetrant (TAP) primate study employed a new topical antipenetrant formulation against *S. mansoni* in *Cebus apella* monkeys.

4. WRHN-III Mb. This prophylactic drug study employed a compound found previously to be highly active against *S. mansoni* in rodents. The compound was evaluated against *S. mansoni* in *Cebus apella* monkeys.

5. WRHN-III Mc. This penetration model study was carried out with *marmoset* monkeys and using *S. mansoni* as the schistosome species.

6. WRHN-IV a. This topical antipenetrant/arthropod repellent compatibility trial in hamsters was carried out to determine what, if any, interactions may exist between TAP and a new arthropod repellent developed by the United States Medical Research and Development Command (USMR&DC). The test was carried out using hamsters exposed to *S. mansoni*.

PROTOCOLS WRHN-II b, WRHN-II c, AND WRHN-IV a
MATERIALS AND METHODS

Techniques for Cultivation and Maintenance of Snail Intermediate Host Species

The procedures used for cultivating and maintaining the species and strains of schistosomes and their respective snail intermediate hosts are described and presented in appendix I.

Animals

The mice used were male ICR (outbred) weighing between 17 and 25 grams. The golden hamsters used were males weighing between 75 and 90 grams.

Schistosome Species

The species of schistosomes used in these protocols were *Schistosoma mansoni* of Puerto Rican and Kenyan origin, *S. haematobium* of Egyptian and Nigerian origin,

and *S. japonicum* of Philippine origin. The snail intermediate host for *S. mansoni* is *Biomphalaria glabrata* from Puerto Rico and *B. sudanica* from Kenya; for *S. haematobium* the snail intermediate host is *Bulinus truncatus truncatus* from Egypt and *B. truncatus rohlfsi* from Nigeria; and for *S. japonicum* the snail intermediate host is *Oncomelania hupensis quadrasi* from the Philippine Islands.

Compounds

Protocol WRHN-II b

A total of ten compounds were received for evaluation in the secondary prophylactic test system. Nine of these drugs were designated as experimental compounds, and one as the positive reference control drug (niridazole). These compounds were selected for advanced testing from the test results of the Brazil schistosomiasis primary prophylactic test and Walter Reed Institute of Research, Division of Experimental Therapeutics in-house testing program. In table 1, the ten compounds tested in rodents are presented by bottle number, Walter Reed accession number, and quantities received at this institution.

Protocol WRHN-II c

A topical antipenetrant (TAP), compound No. BL23702, was received in liquid form and evaluated in hamsters.

Protocol WRHN-IV a

An arthropod-repellent was received in liquid form and was evaluated in conjunction with the topical antipenetrant (BL23702) for possible interactions during future simultaneous use of the two compounds by military personnel.

In regard to the compounds received for testing, there were no known adverse effects involved in handling these substances. However, good laboratory practice has been used. All compounds are stored according to the labeling instructions:

Red label = hygroscopic
Blue label = refrigerate
Green label = compound in liquid form

No label indicates that no special storage conditions are required.

Table 1: Compounds Received at the Center for Tropical Diseases

Bottle Number	Walter Reed Number	Quantity
AQ21946	69504 DO	0.720 grams
BH30042	234928 AA	0.875 grams
BK98839	253817 AA	0.595 grams
BL07459	254583 AA	0.260 grams
BL07468	254574 AA	0.318 grams
BL07566	254581 AA	0.278 grams
BL24503	255967 AA	0.595 grams
BL21459	255751 AA	0.295 grams
BL21468	255752 AA	0.294 grams
BL26758 (niridazole)	005950 AL	3.500 grams

Vehicles

One vehicle was used. Tween 80-methyl cellulose-saline (TMCS) for *per os* treatment. TMCS is made as indicated in table 2.

Table 2: Tween 80-methyl cellulose-saline

Ingredient	Amount
Tween	0.40 ml
Methyl cellulose	0.20 gr
Sodium chloride	0.85 gr
Distilled water	98.55 ml

The sodium chloride was dissolved into distilled water and warmed to 70°C. Five ml of the warm saline solution was added to the Tween 80 and stirred until dissolved. The rest of the saline solution was then added. The solution was allowed to come to room temperature and the methyl cellulose added. The mixture was placed in a refrigerator overnight to facilitate dissolution of the methyl cellulose.

Drug Solutions

All animals were given 100 mg/kg of body weight for *per os* treatments. To obtain the proper concentrations of drug in a reasonable volume of carrier, the following formula was used:

1. 10 mg of compound per ml of carrier (weight per volume)
2. Animal weight (in grams) x 0.01 = ml of solution per animal per day

To calculate the volume of drug solution needed for each day of treatment, the formula below was used:

Number of Animals	x	Average weight plus 10%	x	0.01 ml per gm body weight	=	Total volume used for treatment

Examples: (1) 10 mice x (25 gm + 2.5 gm) x 0.01 ml = 2.75 ml of solution

(2) 10 hamsters x (90 gm + 9 gm) x 0.01 ml = 9.98 ml of solution

To example 1, 27.5 mg of compound is added to 2.75 ml of carrier solution (weight/volume) and to example 2, 99.0 mg of compound is added to 9.9 ml of carrier solution (weight/volume).

Treatment

Per os

Each animal was administered the appropriate volume of drug by use of a garage needle for five consecutive days.

Skin applications

The topical antipenetrant compound was received in liquid form and was applied to previously shaven animal skin (abdominal area) by use of gauze.

Exposures

Mice and hamsters were anesthetized with sodium pentobarbital and exposed percutaneously to numbers of cercariae as shown in tables 3, 4, and 5 (see appendix).

For protocol WRHN-II b, the experimental design is shown in table 3. The experiments were conducted using ten animals for each compound, ten animals as vehicle controls, ten as infection controls (no treatment), and ten as reference drug control (niridazole). Animals were treated on Monday, Tuesday, Wednesday, Thursday, and Friday. All animals were exposed to cercariae on Wednesday, but prior to treatment. Treatment consisted of 100 mg/kg of drug for five consecutive days. Animals were sacrificed and perfused for worms at 49 days after exposure to cercariae.

Table 3: Protocol WRHN-II b Prophylactic Trials in Rodents

Exp. No.	Animal Host	Schistosome Species	No. Cercariae/ Animal	Route of drug Administration	Day of Sacrifice
15	Mouse	*S. mansoni*	150	Oral	49
16	Mouse	*S. mansoni*	150	Oral	49
17	Mouse	*S. mansoni*	150	Oral	49

For protocol WRHN-II c, the experimental design is shown in table 4. A total of thirty animals were used for each species of schistosome studied. The group designations were as follows: ten animals for the topical antipenetrant (TAP), ten for placebo, and ten for infection control group at each time interval, -1, -3, and -7 days, respectively. Animals of the group receiving the topical antipenetrant or placebo solution (see table 4) were treated by applying the drug to the shaven abdominal skin at either -1, -3, or -7 days before exposure to cercariae. The test compounds were applied to animal skin by use of a 2" x 2" gauze pad and wiped on the abdominal area from anterior to anterior position, then left to right. This pattern was repeated for 60 seconds, keeping the treated area wet with solution at all times. After the applied solutions had dried, the abdomens of the animals were washed for one hour with running tap water, 25 to 27 cycles (see figures 1 and 2). The infection control animals (did not receive drug or placebo solution) were also washed.

Table 4: Topical Antipenetrant Trails in Rodents[1]

Schistosome Species	Animal[2] Host	Group Designations	Day of Rx Before Exposure
S. mansoni	Hamster	Topical placebo I.C.[3]	-1, -3, -7
S.haematobium	Hamster	Topical placebo I.C.	-1, -3, -7
S. japonicum	Mouse	Topical placebo I.C.	-1, -3, -7

[1]Day of sacrifice for *S. mansoni* infected animals = 49 days; for *S. japonicum* = 49 days; and for *S. haematobium* = 110 days.
[2]All animals exposed to 240 cercariae.
[3]I.C. = Infection Control.

For protocol WRHN-IV a, the experimental design is presented in table 5. The purpose of this study was to determine what, if any, interactions there might be between the topical antipenetrant and a new improved arthropod repellent in developmental stages. To determine the compatibility of the two compounds, tests were conducted using the golden hamster as the animal test model. In this study, two scenarios were examined. In the first, the arthropod repellant was applied, followed by an application of the topical antipenetrant or the placebo one hour later. In the second scenario, the applications were reversed. In each case, the animals were exposed to *S. mansoni* 24 hours after the last application. A topical antipenetrant control group, arthropod repellant control group, and an infection control group were also included in the study. No washings (as was done in previous topical antipenetrant trials) were done in this study.

Table 5: Topical Antipenetrant/Arthropod Repellent Compatibility Trial in Hamsters

Exp.* Group	First Treatment	Time Interval	Second Treatment	Time Interval	Expose
1	Repellent	1 hour	Topical	24 hours	X
2	Repellent	1 hour	Placebo	24 hours	X
3	Topical	1 hour	Repellent	24 hours	X
4	Placebo	1 hour	Repellent	24 hours	X
5			Topical	24 hours	X
6			Repellent	24 hours	X
7	Infection Control Group				X

*Exp. = Experimental

Recovery of Adult Worms

After the worms had a chance to mature (49 days for both *S. mansoni* and *S. japonicum*, and 90 days for *S. haematobium*), but before the egg burden caused mortality, the animals were sacrificed by injection with 0.5 ml of sodium pentobarbital (65 mg/ml sodium pentobarbital). The animals were necropsied and perfused using a method similar to the Perf-O-Suction method of Radke et al. [17]. The number of male, female, and immature worms were counted and recorded for each animal.

The number of worms recovered from the infected control animals was recorded and used to calculate the relative protection of the antipenetrant using the following formula [18]:

$$\text{Relative Protection} = \frac{x - y}{x} \times 100$$

- Where x = Average number of worms recovered from control animals.
- Where y = Average number of worms recovered from protected animals.

RESULTS

Protocol WRHN-II b

The number of worms recovered at necropsy for animals treated with each of the nine experimental and one reference drug (niridazole) and those of respective control groups is shown in table 6.

The nine experimental compounds studied in this protocol did not provide complete protection against cercarial invasion of any of the mice exposed of any experimental group. Mature active male and female worms and viable eggs in tissue were observed for all groups of mice treated with either of the test compounds. The

reference compound (niridazole) did not provide protection against this strain of *S. mansoni* from Kenya. This is in contrast to the activity of this compound against other strains of *S. mansoni*.

Of the compounds evaluated for prophylactic activity against *S. mansoni* (Kenyan strain) in this study, one of them (BH30042) showed a level of protection of 92%. However, there were small numbers of mature active male and female worms as well as viable eggs in tissue. The other eight compounds showed very low levels of protection with mature active male and female worms and viable eggs in the tissue.

Table 6: Efficacy of Prophylactic Oral Doses of Test Compounds Against *Schistosoma mansoni* (Kenyan Strain) in Mice Exposed Experimentally to 150 Cercariae

Experiment #	Control/Drug Group	Worm Burdens After Perfusion: Collection Filter[1] M[3]	F[4]	SM[5]	SF[6]	Tissue Examination[2] M	F	SM	SF	Total No. Worms	Animals	Efficacy (%)
15	Untreated	270	254	15	259	3	4	0	4	809	10	---
	TMCS (vehicle)	269	243	3	213	2	2	2	3	737	10	---
	BL26758 (RC[7])	176	151	10	27	3	2	1	1	371	10	49.7
	ZP55645	277	266	32	194	2	3	1	0	775	10	-5.2
	BL07459	277	246	6	220	0	0	0	0	749	10	-1.6
	AQ21946	268	269	9	148	2	1	1	0	698	9	-5.3
16	Untreated	482	283	21	2	0	1	0	0	789	10	---
	TMCS (vehicle)	311	265	0	52	0	0	0	0	628	9	---
	BL26758 (RC)	110	66	10	7	0	0	0	0	193	10	72.3
	BK98839	456	360	5	26	1	1	0	0	849	10	-21.6
	BL24503	489	395	1	1	3	3	0	0	892	10	-27.8
	BL07566	464	348	3	1	8	10	0	0	834	10	-19.5
17	Untreated	221	196	0	0	1	0	0	0	418	9	---
	TMCS (vehicle)	228	209	0	0	0	0	0	0	437	10	---
	BL26758 (RC)	164	92	0	0	6	4	0	0	266	10	39.1
	BH30042	22	11	0	0	0	0	0	0	33	10	92.4
	BL078468	233	213	8	61	0	0	0	0	515	10	-17.8
	BL55636	257	229	10	57	0	0	0	0	553	10	-26.5

[1]Filter used to trap worms perfused from the liver and mesenteric veins.
[2]Examinations of liver, mesenteric veins, and adipose tissue containing veins for lodged worms after perfusion.
[3]Male worms.
[4]Female worms.
[5]Stunted male worms.
[6]Stunted female worms.
[7]Reference Compound (Niridazole).

Protocol WRHN-II c

The efficacy of the topical antipenetrant against three species of schistosomes infective to humans is shown in tables 7 through 10. The number of worms

recovered at necropsy for each animal treated at either -1, -3, or -7 days before exposure and their respective control groups is shown in tables 7 through 10.

The efficacy of the test compound against drug-resistant (oxamniquine-resistant) *S. mansoni* cercariae of Brazilian (MAP strain) origin is shown in table 7. Complete protection (100%) was obtained for animals receiving the drug at -1 day before exposure to cercariae. Complete protection was not obtained for any of the animals treated at -3 or -7 days before exposure to cercariae. The efficacies of the drug for these groups varied from 68% (treated at -3 days before exposure) and 12% (treated at -7 days before exposure). The corresponding control groups (placebo and infection control) animals were all infected with large numbers of mature adult worms. Gross pathology indicative of urinary schistosomiasis was observed in all control groups as well as in the two groups of animals treated at -3 and -7 days before exposure to cercariae.

Table 7: Prophylactic Effect of the Topical Antipenetrant BL44970 Against *Schistosoma mansoni* (MAP-Brazilian Strain) in Hamsters Exposed to 240 Cercariae

Worm Burdens After Perfusion											
Control/Drug Group	Collection Filter[1]				Tissue Examination[2]				Total		Efficacy (%)
	M[3]	F[4]	SM[5]	SF[6]	M	F	SM	SF	Worms	Animals	
IC[7] - 0 day	327	331	4	45	30	31	0	0	768	9	---
IC - 1 day	144	115	0	0	4	5	0	0	268	5	---
IC - 3 days	205	186	1	0	8	9	0	0	409	5	---
IC - 7 days	186	161	1	0	15	17	0	0	380	5	---
BL44989 - 1 day (Placebo)	287	242	0	22	6	5	0	0	562	10	---
BL44989 - 3 days	327	277	9	13	12	11	0	0	649	10	---
BL44989 - 7 days	325	310	0	52	13	13	0	0	713	10	---
BL44970 - 1 day (Niclosamide)	0	0	0	0	0	0	0	0	0	10	100
BL44970 - 3 days	96	70	0	24	8	8	0	0	206	10	68
BL44970 - 7 days	297	266	1	33	16	17	0	0	630	10	12

[1]Filter used to trap worms perfused from the liver and mesenteric veins.
[2]Examinations of liver, mesenteric veins, and adipose tissue containing veins for lodged worms after perfusion.
[3]Male worms.
[4]Female worms.
[5]Stunted male worms.
[6]Stunted female worms.
[7]Infection Control.

The efficacy of the test compound against drug-resistant (niridazole-resistant) *S. mansoni* cercariae of Kenyan origin is presented in table 8. Complete protection (100%) was obtained for animals receiving the drug at -1 day before exposure to cercariae. Animal receiving the drug at -3 and -7 days before exposure to cercariae were not completely protected. The efficacies for these groups were 86% and 18% respectively. Animals of the corresponding control groups (placebo and infection controls) were all highly infected with large numbers of active mature male and female worms. The gross pathology observed in the control groups as well as in the two groups treated at -3 and -7 days before exposure was indicative of intestinal schistosomiasis.

Table 8: Prophylactic Effect of the Topical Antipenetrant BL44970 Against *Schistosoma mansoni* (Kenyan Strain) in Hamsters Exposed to 240 Cercariae

Worm Burdens After Perfusion											
Control/Drug Group	Collection Filter[1]				Tissue Examination[2]				Total		Efficacy (%)
	M[3]	F[4]	SM[5]	SF[6]	M	F	SM	SF	Worms	Animals	
IC[7] - 0 day	185	176	1	60	3	3	0	0	428	10	---
IC - 1 day	159	152	2	9	6	6	0	0	334	5	---
IC - 3 days	156	164	0	22	5	6	0	0	353	5	---
IC - 7 days	150	124	0	0	0	1	0	0	275	5	---
BL44989 - 1 day (Placebo)	292	278	3	52	10	12	0	0	647	10	---
BL44989 - 3 days	268	252	1	16	9	7	0	0	553	10	---
BL44989 - 7 days	249	237	0	120	6	3	0	0	615	10	---
BL44970 - 1 day (Niclosamide)	0	0	0	0	0	0	0	0	0	10	100
BL44970 - 3 days	34	29	0	5	3	4	0	0	75	10	86
BL44970 - 7 days	204	205	4	80	5	5	0	0	503	10	18

[1]Filter used to trap worms perfused from the liver and mesenteric veins.
[2]Examinations of liver, mesenteric veins, and adipose tissue containing veins for lodged worms after perfusion.
[3]Male worms.
[4]Female worms.
[5]Stunted male worms.
[6]Stunted female worms.
[7]Infection Control.

The efficacy of the test company against *S. japonicum* cercariae of Philippine origin is presented in table 9. No complete protection was obtained when the topical antipenetrant was used against the cercariae of *S. japonicum* (Philippine strain) in mice. The levels of protection observed were very low, ranging from 42.7% for

animals treated at -1 day before exposure, 39.2% for those treated at -3 days, and 12.3% for those treated at -7 days before exposure to cercariae respectively.

Table 9: Prophylactic Effect of the Topical Antipenetrant BL44970 Against *Schistosoma japonicum* (Philippines) in Mice Exposed to 30 Cercariae

Worm Burdens After Perfusion											
Control/Drug Group	Collection Filter[1]				Tissue Examination[2]				Total		Efficacy (%)
	M[3]	F[4]	SM[5]	SF[6]	M	F	SM	SF	Worms	Animals	
IC[7] - 0 day	121	88	26	0	0	0	0	0	235	10	---
IC - 1 day	59	58	21	1	1	1	0	0	141	5	---
IC - 3 days	58	55	2	0	0	0	0	0	115	5	---
IC - 7 days	65	50	0	0	0	0	0	0	115	5	---
BL44989 - 1 day (Placebo)	136	120	10	1	0	0	0	0	267	10	---
BL44989 - 3 days	144	105	0	1	0	0	0	0	250	10	---
BL44989 - 7 days	117	115	9	19	0	0	0	0	260	10	---
BL44970 - 1 day (Niclosamide)	58	52	0	42	0	1	0	0	152	10	42.7
BL44970 - 3 days	71	61	1	4	0	0	0	0	137	9	39.2
BL44970 - 7 days	99	91	38	0	0	0	0	0	228	10	12.3

[1]Filter used to trap worms perfused from the liver and mesenteric veins.
[2]Examinations of liver, mesenteric veins, and adipose tissue containing veins for lodged worms after perfusion.
[3]Male worms.
[4]Female worms.
[5]Stunted male worms.
[6]Stunted female worms.
[7]Infection Control.

The efficacy of the test compound against *S. haematobium* of Egyptian origin in shown in table 10. Nearly complete (98.9%) protection was obtained for animals treated at -1 day before exposure to cercariae, and at -3 days before exposure, the level of protection was 98.3% for animals of this group. For the animals of the group treated at -1 before treatment, only two (one male and one female) adult worms were recovered, while among the animals treated at -3 days before exposure, four male worms were recovered. The level of protection observed for animals of the group treated at -7 days before treatment was much lower, that is, 23.3%. Animals of the corresponding control group and the placebo group were all heavily infected with active mature male and female worms. The gross pathology observed in these two groups, as well as in the group treated at -7 days before exposure, was indicative of urinary schistosomiasis.

Table 10: Prophylactic Effect of the Topical Antipenetrant BL44970 Against *Schistosoma haematobium* (Egyptian Strain) in Hamsters Exposed to 240 Cercariae

Worm Burdens After Perfusion											
Control/Drug Group	Collection Filter[1]				Tissue Examination[2]				Total		Efficacy (%)
	M[3]	F[4]	SM[5]	SF[6]	M	F	SM	SF	Worms	Animals	
IC[7] - 0 day	139	76	0	3	3	2	0	0	223	10	---
IC - 1 day	13	7	0	0	3	4	0	0	27	1	---
IC - 3 days	64	48	0	1	7	8	0	0	128	5	---
IC - 7 days	77	48	0	2	0	0	0	0	127	5	---
BL44989 - 1 day (Placebo)	94	58	3	2	15	15	0	0	187	10	---
BL44989 - 3 days	128	99	0	1	2	2	0	0	232	10	---
BL44989 - 7 days	110	63	1	0	1	1	0	0	176	10	---
BL44970 - 1 day (Niclosamide)	0	0	0	0	1	1	0	0	2	10	98.9
BL44970 - 3 days	4	0	0	0	0	0	0	0	4	10	98.3
BL44970 - 7 days	63	39	0	0	3	3	0	0	108	8	23.3

[1]Filter used to trap worms perfused from the liver and mesenteric veins.
[2]Examinations of liver, mesenteric veins, and adipose tissue containing veins for lodged worms after perfusion.
[3]Male worms.
[4]Female worms.
[5]Stunted male worms.
[6]Stunted female worms.
[7]Infection Control.

Protocol WRHN-IV a

The result of the study to determine if interactions occur between the topical antipenetrant (TAP) and the arthropod repellent when applied to skin of hamsters at 24 hours before exposure to a drug-resistant (oxamniquine-resistant) strain of *S. mansoni* cercariae originating from Brazil is shown in table 11.

When the topical antipenetrant was applied to hamster skin alone or in combination (TAB/repellant or repellant/TAP) with the repellent at one-hour intervals, complete protection (100%) was obtained. When the repellent was applied alone, approximately 50% of the invading cercariae failed to develop. Use of the repellent in combination (placebo/repellent or repellent/placebo) did not significantly affect the development of cercariae to mature adult worms. Gross pathology observed for infection controls animals and for those receiving the repellent alone or in combination with the placebo was indicative of intestinal

schistosomiasis. All of the worms recovered from these groups of animals were mature male and female schistosomes.

Table 11: Compatibility Studies Between a Topical Antipenetrant (TAP) and Arthropod-Repellent in Hamsters Exposed to 240 *S. mansoni* (MAP-Brazilian Strain) Cercariae

Worm Burdens After Perfusion											
Control/Drug Group	Collection Filter[1]				Tissue Examination[2]				Total		Efficacy (%)
	M[3]	F[4]	SM[5]	SF[6]	M	F	SM	SF	Worms	Animals	
IC[7]	228	224	1	64	6	6	0	0	529	10	---
Repellent	140	121	0	19	0	0	0	0	280	10	47.1
TAP (BL44970)	0	0	0	0	0	0	0	0	0	10	100
Placebo (BL44989)/ Repellent	206	188	0	28	3	3	0	0	428	9	10.2
TAP/Repellent	0	0	0	0	0	0	0	0	0	10	100
Repellent/ Placebo	251	234	0	90	6	7	0	0	588	10	-11.2
Repellent/TAP	0	0	0	0	0	0	0	0	0	10	100

[1]Filter used to trap worms perfused from the liver and mesenteric veins.
[2]Examinations of liver, mesenteric veins, and adipose tissue containing veins for lodged worms after perfusion.
[3]Male worms.
[4]Female worms.
[5]Stunted male worms.
[6]Stunted female worms.
[7]Infection Control.

PROTOCOL WRHN-III Ma
BACKGROUND

The topical antipenetrant (TAP) compound which was evaluated in this protocol was previously evaluated in mice, hamsters, and rhesus monkeys and provided virtually 100% protection against skin invasion by the cercariae of *S. mansoni*, *S. japonicum*, and *S. haematobium*. As a result of the excellent results obtained previously, a final formulation was prepared for possible studies in human volunteers. However, before this phase of study could be initiated, the efficacy of the formulation was tested in mice and hamsters, and then in monkeys. This protocol covers the final evaluation of the formulation in *Cebus apella* monkeys.

MATERIALS AND METHODS

Test Compound

The topical antipenetrant formulation (BL44970) and the placebo (BL44989) were prepared by Miles Laboratory through an agreement with the Department of Medicinal Chemistry, Division of Experimental Therapeutics, Walter Reed Army Institute of Research. Both the test compound and the placebo were prepared in the same vehicle.

Animals

Cebus apella were obtained from Worldwide Primates, Inc., Miami, Florida. Monkeys were kept under quarantine for 45 days at Worldwide facilities prior to shipment to Lowell. Upon arrival at the University of Lowell, they were immediately placed in quarantine for 45 days during which time they were tested for tuberculosis by the skin test method and examined for helminthic infections. Both males and females weighing between 2.5—4.5 kg were used.

Animals were housed individually in standard regulation cages, fed monkey chow and water *ad libitum*. All animals are kept in a temperature/humidity controlled animal quarters in a twelve-hour light/dark cycle.

Animal Care

Monkeys are maintained as outlined by the "Guide for the Care and Use of Laboratory Animals" (1986).

All animals were anesthetized with intramuscular injections of a mixture of ketamine hydrochloride to temporarily immobilize them during treatment applications, washing, and exposure to parasites. No painful or stressful treatments occurred.

Monkeys were sacrificed at the termination of the study by giving an overdose of the anesthetic and exsanguination.

Methods

Animals were treated, washed, exposed, and examined according to the study design outlined in table 12.

Table 12: Experimental Design for the *Schistosoma mansoni* Antipenetrant Study (BL44970) in *Cebus apella* Monkeys

Group No.	Days Treated Pre-Infection	Treatment Type	No. of Animals
I	7	1% BL44970	4
II	7	Placebo	2
III	3	1% BL44970	4
IV	3	Placebo	2
V	1	1% BL44970	4
VI	1	Placebo	2
VII	0	Infection Control	5

Total number of animals = 23.

Drug Application

Animals were anesthetized as described, and barber's clippers were used to remove the coarse hair from the entire right arm of each animal. The drug solution was applied to the clipped arms of the experimental animals using a 4 x 4" gauze pad (clamped by and wrapped around a hemostat). The entire arm was treated by wiping it from the shoulder and down the arm to include the hands. Vehicle control animals were treated in the same manner with the placebo.

Note: 20 ml aliquots of the drug solution and placebo were allotted per animal. The total volume was not used for each animal. The amount used for each animal was that volume required to cover the previously shaved area of the right arm.

Washing

For all experimental animals, including the placebos, topically treated areas were washed 10 minutes post-treatment to remove excess compound which had not been topically absorbed. Each treated arm was placed in a 152 x 450 mm pipet washer and washed with filtered water at one cycle per minute for 30 minutes (one cycle is defined as the amount of water filling from the level of the finger tips to the shoulder and draining from the shoulder to the finger tips).

Exposure

An oxamniquine-resistant strain of *S. mansoni* known as MAP which was obtained from Brazil in January 1986, was used for all schistosome infections. This strain is maintained in laboratory bred *Biomphalaria glabrata* (BH-Belo Horizonte, State of Minas Gerais, Brazil) and outbred CD_1 albino male mice. Cercariae were shed from infected *B. glabrata* en masse as follows:

1. The evening prior to the days of exposure, the trays containing the infected snails were covered with a black cloth to simulate a dark period.

2. Infected *B. glabrata* were stressed by drying them on paper towels and exposing them to fluorescent light for one-half hour.

3. Filtered, aerated water was then added to the beaker containing the infected snails to just cover the snails.

4. The beaker was observed for shedding.

5. The first addition of water was then discarded to remove dirt, fecal matter, etc., and a second addition of water was made to collect the cercariae.

6. The solution containing cercariae was poured into a petri dish and used for the infection of experimental animals.

7. The cercariae were counted by drawing them from the solution with a micro-pipet as observed through a dissecting microscope.

Note: when necessary, the cercarial solution was diluted with filtered, aerated water to facilitate ease in counting the correct number of cercariae.

The *Cebus apella* monkeys were exposed to a dose of 400 cercariae each for 45 minutes. Ten CD_1 Swiss albino male mice were exposed to 100 cercariae each by the tail immersion method for 45 minutes in order to determine the viability of the cercariae. Five mice were exposed prior to the exposure of the experimental animals. The remaining five mice were exposed following exposure of the monkeys.

The monkeys were anesthetized as previously described for drug treatment. The treated arms of monkeys were immersed into a 1.4 liter Rubbermaid refrigerator door pitcher as shown in photographs 3 and 4. The water level in the containers was such that it was below the uppermost washed area of the forearm. The monkeys were exposed for 45 minutes, and the arms were then drawn from the container and allowed to air dry. Following exposure of the monkeys, 100 ml aliquots of formalin were added to the exposure solution. The contents of the containers were concentrated by pouring the infection solution through a 20μ sieve (3" in diameter). The recovered cercariae were washed off the sieve with filtered water into a petri dish. The cercariae were then stained with 4% Lugol's iodine for counting. The number of cercariae remaining in exposure containers after exposure of each monkey and control mouse is shown in tables 13 and 14.

Table 13: Result of Examination of Exposure Containers for Cercariae After Exposure of Monkeys

Group #	Cercarial Count	% of Cercariae in Exposure Containers	% of Intact Cercariae	Group
1	190	48	6	
2	274	69	75	7A
3	132	33	39	
4	100	25	58	
5	180	45	76	7B
6	140	35	81	
7	215	54	20	
8	186	47	61	3A
9	66	17	73	
11	266	67	63	
12	231	58	73	3B
14	105	26	94	
15	241	60	27	
19	303	76	75	1A
20	192	48	92	
21	367	92	72	
22	336	84	42	1B
23	83	21	89	
24	102	26	54	Infection
25	96	24	80	Control-A
30	117	29	84	Infection
31	81	20	73	Control-B
32	113	28	72	

[1]All "A" groups were exposed on 16 July 1987; all "B" groups were exposed on 17 July 1987.
[2]On 16 July 1987, the monkeys were exposed in the following order: 1, 24, 3, 7, 9, 15, 2, 8, 19, 20, 25. On 17 July 1987, the monkeys were exposed in the following order: 30, 4, 11, 14, 21, 23, 31, 5, 6, 12, 22, 32.
[3]All monkeys were exposed to 400 cercariae each.

Table 14: Results for Mice Exposed on 16 and 17 July 1987

Number	Cercarial Count for Those Exposed 16 July 1987	Cercarial Count for Those Exposed 17 July 1987
1	0	0
2	0	0
3	0	0
4	1	0
5	2	0
6	0	0
7	3	0
8	4	0
9	4	1
10	5	4

Animals 1-5 were exposed prior to exposure of the first monkey; animals 6-10 were exposed after exposure of the last monkey.

All equipment which may have been contaminated with cercariae was disinfected with bleach (5.25% sodium hypochlorite). All personnel required to handle cercariae were protected from accidental infection by disposable protective garments, disposable surgical gloves, and full face visors. Bleach (5.25% sodium hypochlorite) and 95% ethanol were available at all times during the infection to decontaminate any accidental spills or equipment or personnel respectively.

Examination

Prior to the beginning of experimentation, all monkeys were screened for parasites. Fecal samples were examined using the AMS-3 concentration technique.

Beginning on day 28 post-exposure and continuing twice per week until the termination of the study, fecal samples from each animal were examined for schistosome eggs using the AMS-3 concentration technique.

All animals were sacrificed and examined 49-54 days post-exposure to *S. mansoni*. Any schistosome (mature and/or stunted) parasites present were recovered from each animal by the Perf-O-Suction technique, counted, and the efficacy of the drug computed.

RESULTS

The number of cercariae remaining in monkey exposure containers after 45 minutes of contact with the water containing the infective schistosome larva (cercariae) is presented in table 13. The percentages of cercariae (intact and non-intact) ranged from 92% to 17% depending on the particular group of monkeys. The lowest percentages of cercariae remaining in the exposure containers were obtained for the infection control group of monkeys which did not receive any drug application to their skin.

The number of cercariae remaining in mouse exposure chambers was very low for both groups of control mice (table 14) as compared to the numbers of cercariae remaining in the monkey exposure units (table 13).

The efficacy of the topical antipenetrant (TAP) formulation against drug-resistant (oxamniquine-resistant) cercariae of Brazilian (MAP-strain) origin is shown in table 15. Complete protection was obtained for animals receiving the drug application at -1 and -3 days before exposure to cercariae. For the group of animals receiving the drug application -7 days before exposure, two of the animals were completely protected, while the remaining two animals had levels of protection at

78.9% and 91.0% respectively. Mature adult worms (14 and 6 respectively) were recovered, and viable eggs were obtained in organ tissue.

Table 15: Prophylactic Effect of a Topical Antipenetrant (TAP) BL44970 Against *Schistosoma mansoni* (MAP-Brazilian Strain) in *Cebus apella* Exposed Experimentally to 400 Cercariae

Worm Burdens After Perfusion												
Control/Drug Group	Animal		Collection Filter[1]				Tissue Examination[2]				Total No. Worms	Efficacy (%)
	No.	Sex	M[3]	F[4]	SM[5]	SF[6]	M	F	SM	SF		
Infection Control	24	F	38	24	—	—	15	16	—	—	93	—
	25	M	19	11	—	—	16	15	—	—	61	—
	30	F	7	6	—	1	21	22	—	2	59	—
	31	F	6	6	—	—	3	3	—	—	18	—
	32	F	39	43	—	—	10	13	—	—	105	—
BL44989 (Placebo) 1 Day	20	F	10	8	—	—	—	—	—	—	18	—
	23	F	9	3	—	—	5	5	—	—	22	—
BL44989 (Placebo) 3 Days	9	M	52	58	—	—	12	12	—	—	134	—
	14	F	16	26	—	—	6	5	—	—	53	—
BL44989 (Placebo) 7 Days	3	M	13	12	—	—	1	1	—	—	27	—
	6	M	37	38	—	—	15	16	—	—	106	—
BL44970 (Test-Compound) 1 Day	15	F	—	—	—	—	—	—	—	—	0	100
	19	M	—	—	—	—	—	—	—	—	0	100
	21	F	—	—	—	—	—	—	—	—	0	100
	22	F	—	—	—	—	—	—	—	—	0	100
BL44970 (Test-Compound) 3 Days	7	M	—	—	—	—	—	—	—	—	0	100
	8	F	—	—	—	—	—	—	—	—	0	100
	11	M	—	—	—	—	—	—	—	—	0	100
	12	F	—	—	—	—	—	—	—	—	0	100
BL44970 (Test-Compound) 7 Days	1	M	—	—	—	—	—	—	—	—	0	100
	2	M	—	—	—	—	—	—	—	—	0	100
	4	F	8	6	—	—	—	—	—	—	14	78.9
	5	M	1	3	—	—	1	1	—	—	6	91.0

[1]Filter used to trap worms perfused from the liver and mesenteric veins.
[2]Examinations of liver, mesenteric veins, and adipose tissue containing veins for lodged worms after perfusion.
[3]Male worms.
[4]Female worms.
[5]Stunted male worms.
[6]Stunted female worms.

All animals of the infection control and placebo groups were found to be infected with male and female adult worms. The worm burden levels observed for each of these groups of controls were very similar both in individual ranges and overall group averages. Each of these animals had viable eggs in their organ tissues, and nearly all worms were mature adults.

PROTOCOL WRHN-III Mb

BACKGROUND

Compound BL23702 (WR249313) was previously found to be highly active as an oral prophylactic compound against *Schistosoma mansoni* in infected mice. The compound was evaluated in this protocol as an oral prophylactic in *Cebus apella* monkeys.

MATERIAL AND METHODS

Test Compound

BL23702 (WR249313) was obtained from the Department of Medicinal Chemistry, Division of Experimental Therapeutics, Walter Reed Army Institute of Research. The vehicle used for preparation of the compound was Tween 80-methyl cellulose saline (TMCS).

Animals

Cebus apella were obtained from Worldwide Primates, Inc., Miami, Florida. Monkeys were kept under quarantine for 45 days at Worldwide facilities prior to shipment to Lowell. Upon arrival at the University of Lowell, they were immediately placed in quarantine for 45 days during which time they were tested for tuberculosis by the skin test method and examined for helminthic infections. Both males and females weighing between 2.5—4.5 kg were used.

Animals were housed individually in standard regulation cages, fed monkey chow and water *ad libitum*. All animals are kept in a temperature/humidity controlled animal quarters in a twelve-hour light/dark cycle.

Animal Care

Monkeys are maintained as outlined by the "Guide for the Care and Use of Laboratory Animals" (1986).

All animals were anesthetized with intramuscular injections of a mixture of ketamine hydrochloride to temporarily immobilize them during treatment applications, washing, and exposure to parasites. No painful or stressful treatments occurred.

Monkeys were sacrificed at the termination of the study by giving an overdose of anesthesia folowed by exsanguination.

Methods

Animals were treated, washed, exposed, and examined according to the study design outlined in table 16.

Table 16: Experimental Design for the *Schistosoma mansoni* Oral Prophylactic Study (BL23702) in *Cebus apella*

Group	Treatment Type	No. of Animals
I	Infection Control	4
II	Vehicle Control[1]	2
III	Reference Drug[2]	2
IV	Test Drug[3]	6

[1]Tween 80-methyl cellulose saline (TMCS)
[2]BL26758 (Niridazole)
[3]BL23702 (BL26450)

Drug Administration

Monkeys were given the drug while under slight anesthesia via a stomach tube. Each animal to be treated receive the drug orally at a dosage of 100 mg/kg for five days. The treated animals were observed for signs of gross toxicity up to at least four hours post-therapy.

Exposure

An oxamniquine-resistant strain of *S. mansoni* known as MAP and obtained from Brazil in January 1986, was used for all schistosome infections. This strain is maintained in laboratory bred *Biomphalaria glabrata* (BH-Belo Horizonte, State of Minas Gerais, Brazil) and outbred CD_1 albino male mice. Cercariae were shed from infected *B. glabrata* en masse as follows:

1. The evening prior to the day of exposure, the trays containing the infected snails were covered with a black cloth to simulate a dark period.
2. Infected *B. glabrata* were stressed by drying them on paper towels and exposing them to fluorescent light for one-half hour.
3. Filtered, aerated water was then added to the beaker containing the infected snails to just cover the snails.
4. The beaker was observed for shedding.
5. The first addition of water was then discarded to remove dirt, fecal matter, etc., and a second addition of water was made to collect the cercariae.

6. The solution containing cercariae was poured into a petri dish and used for the infection of experimental animals.

7. The cercariae were counted by drawing them from the solution with a micro-pipet as observed through a dissecting microscope.

Note: when necessary, the cercarial solution was diluted with filtered, aerated water to facilitate ease in counting the correct number of cercariae.

Monkeys were anesthetized with intramuscular injections of ketamine, and hamsters which were exposed as controls for cercarial viability were immobilized by intraperitoneal injections of 20% sodium pentobarbital. The abdomens were shaved using animal clippers, then pre-wetted with conditioned water. Cercariae were counted by drawing them from a pool using a micro-pipet as observed through a dissecting microscope and transferred directly to the abdomens of the animals. In the case of the monkeys, the cercariae were concentrated in a specific area by placing a handmade glass tube with a flared base (1.8 cm in diameter x 2.5 cm in height) on the abdomens and expelling the cercariae into it. All animals were exposed to cercariae for 30 minutes.

Examination

Prior to the beginning of experimentation, all monkeys were screened for parasites. Fecal samples were examined using the AMS-3 concentration technique.

Beginning on day 28 post-exposure and continuing twice per week until the termination of the study, fecal samples from each animal were examined for schistosome eggs using the AMS-3 concentration technique.

All animals were sacrificed and examined 49-54 days post-exposure to *S. mansoni*. Mature adult parasites were recovered from each animal by the Perf-O-Suction technique, counted, and the efficacy of the drug computed.

RESULTS

The efficacy of compound BL23702 against a drug-resistant (oxamniquine-resistant) strain (MAP) of *S. mansoni* of Brazilian origin is presented in table 17. This compound was found to be complete inactive in *Cebus apella* monkeys when administered orally as a prophylactic for five consecutive days. The mature adult male and female worms recovered from the animals receiving the test compound were equal in numbers to those worms recovered from the infection control and vehicle control monkeys. Viable schistosome eggs were found in organ tissue, and

gross pathology was consistent with that observed for active schistosomiasis in experimentally infected monkeys.

Table 17: Efficacy of the Oral Prophylactic Compound BL23702 Against *Schistosoma mansoni* (MAP-Brazilian Strain) in *Cebus apella* Exposed Experimentally to 400 Cercariae

Worm Burdens After Perfusion												
Control/Drug Group	Animal		Collection Filter[1]				Tissue Examination[2]				Total No. Worms	Efficacy (%)
	No.	Sex	M[3]	F[4]	SM[5]	SF[6]	M	F	SM	SF		
Infection Control	8	F	10	6	—	—	45	50	—	—	111	—
	18	M	29	31	—	—	42	45	4	—	151	—
	28	M	25	18	—	—	34	40	—	—	117	—
	29	F	97	85	—	9	9	10	—	—	210	—
TMCS (Vehicle)	17	F	13	13	5	—	29	29	—	—	89	—
	33	F	38	13	—	—	58	61	—	—	181	—
BL23702 (Test-Compound)	3/818	F	38	13	—	—	113	118	—	—	282	-108.9
	10	M	37	33	—	—	37	43	—	—	50	-11.1
	16	F	41	35	—	—	38	39	—	—	153	-13.3
	26	M	36	38	—	—	25	29	—	—	128	5.2
	27	M	7	8	—	5	49	53	—	—	122	9.6
	43/5	F	36	31	-	—	8	8	—	••	83	38.5

Note: The positive drug control group (which consisted of two monkeys) was dropped from the experiment on August 12, 1987, due to drug toxicity.

[1]Filter used to trap worms perfused from the liver and mesenteric veins.
[2]Examinations of liver, mesenteric veins, and adipose tissue containing veins for lodged worms after perfusion.
[3]Male worms.
[4]Female worms.
[5]Stunted male worms.
[6]Stunted female worms.

PROTOCOL WRHN-III Mc
BACKGROUND

The marmoset monkey was investigated as a possible model for the purpose of studying schistosome penetration dynamics. This species apparently has a natural skin barrier to schistosome cercariae, but when cercariae are injected subcutaneously some of the injected cercariae are able to survive and mature into adult worms. This study was designed to confirm these findings and thus provide the foundation for further in-depth studies.

Animals

Marmoset were obtained from Worldwide Primates, Inc., Miami, Florida. Monkeys were kept under quarantine for 45 days at Worldwide facilities prior to shipment to Lowell. Upon arrival at the University of Lowell, they were immediately placed in quarantine for 45 days during which time they were tested for tuberculosis by the skin test method and examined for helminthic infections. Both male and female marmosets weighed about 1.1 kg.

Animals were housed individually in standard regulation cages, fed monkey chow and water *ad libitum*. All animals are kept in a temperature/humidity controlled animal quarters in a twelve-hour light/dark cycle.

Animal Care

Monkeys are maintained as outlined by the "Guide for the Care and Use of Laboratory Animals" (1986).

All animals were anesthetized with intramuscular injections of a mixture of ketamine hydrochloride to temporarily immobilize them during exposure to parasites. No painful or stressful treatments occurred.

Monkeys were sacrificed at the termination of the study by giving an overdose of the anesthesia followed by exsanguination.

METHODS

Animals were treated, washed, exposed, and examined according to the study design outlined in table 18.

Table 18: Experimental Design for Marmoset Experiment

Group	Method of Exposure	No. of Animals	Necropsy*
1	Subcutaneously	4	56 days
2	Percutaneously	4	120 days

*Two monkeys from each group were necropsied at 56 days and two from each group at 120 days post-exposure.

Exposure

An oxamniquine-resistant strain of *S. mansoni* known as MAP and obtained from Brazil in January 1986, was used for all schistosome infections. This strain is maintained in laboratory bred *Biomphalaria glabrata* (BH-Belo Horizonte, State of Minas Gerais, Brazil) and outbred CD_1 albino male mice. Cercariae were shed from infected *B. glabrata* en masse as follows:

1. The evening prior to the days of exposure, the trays containing the infected snails were covered with a black cloth to simulate a dark period.

2. Infected *B. glabrata* were stressed by drying them on paper towels and exposing them to fluorescent light for one-half hour.

3. Filtered, aerated water was then added to the beaker containing the infected snails to just cover the snails.

4. The beaker was observed for shedding.

5. The first addition of water was then discarded to remove dirt, fecal matter, etc., and a second addition of water was made to collect the cercariae.

6. The solution containing cercariae was poured into a petri dish and used for the infection of experimental animals.

7. The cercariae were counted by drawing them from the solution with a micro-pipet as observed through a dissecting microscope.

Note: when necessary, the cercarial solution was diluted with filtered, aerated water to facilitate ease in counting the correct number of cercariae.

Monkeys were anesthetized with intramuscular injections of ketamine, and hamsters which were exposed as controls for the cercarial viability were immobilized by intraperitoneal injections of 20% sodium pentobarbital (1 cc/100 gm of body weight). The abdomens of monkeys and mice were shaved using animal clippers, then pre-wetted with conditioned water. Cercariae were counted by drawing them from a pool using a micro-pipet as observed through a dissecting microscope and transferred directly to the abdomens of the mice, and to a 1 cc syringe for the monkeys. Metal rings (2 cm in diameter x 1 cm in height) were placed on the abdomens of the monkeys. The cercariae were then expelled from the syringe into the metal ring. All animals were exposed to cercariae for 30 minutes.

Examination

Monkeys were paired for sex and weight for matched-pair analysis. Four monkeys were infected subcutaneously and four percutaneously with 150 *S. mansoni* cercariae each. Animals were checked for patency by stool concentration method starting at six weeks post-infection. This was done on a daily basis until evidence of patency occurred.

After patency, a weekly quantitative stool egg count for each monkey was performed. At approximately eight weeks after exposure, four monkeys were sacrificed (two from each group, see table 18).

At necropsy portal pressure was measured, and microspheres were injected in the portal vein. Then animals were perfused by the Perf-O-Suction technique. Major organs were taken and digested with KOH to obtain egg counts for organ egg load analysis. Worms were counted, their sex and condition noted, as well as gross pathology of the organs.

The remaining four monkeys were maintained for another four months after which they were to be sacrificed and the same procedure as described above performed.

RESULTS

Schistosome eggs were found in the feces of the group of animals receiving cercariae via the subcutaneous route on the forty-eighth day after exposure. Thereafter no eggs were ever found in the feces of animals of either group (exposed subcutaneously or percutaneously) for the remainder of the observation period.

Two of the animals of the group receiving cercariae subcutaneously were sacrificed at 73 days after exposure. One animal had 8 (immature) worms while the other animal had 26 (immature) worms.

Based on results obtained for fecal examinations and those obtained at sacrifice of the two animals which showed immature worms, it was decided not to sacrifice the remaining animals as this would constitute unnecessary and unreasonable use of these animals. The experiment was discontinued and the remaining animals sent to the U.S. Army's breeding laboratory for marmosets at Edgewood Arsenal, Maryland.

DISCUSSION

The nine compounds studied under protocol WRHN-II b were priority prophylactic candidate drugs found previously to be highly active in the rodent primary prophylactic test. They were referred for study in the prophylactic test and were studied using an oral prophylactic regimen against *S. mansoni* in mice. At the drug dosages used (100 mg/kg x 5 days), complete protection was not obtained for any of the compounds.

Since all animals examined (including those from groups where 92% protection levels were obtained) harbored mature male and female worms and viable eggs in organ tissue which caused gross pathology consistent with active intestinal schistosomiasis, no further studies are recommended for these compounds.

The new topical antipenetrant (TAP) formulation was found to be as active as a previous formulation when applied to hamster skin at -1 and -3 days before exposure to either *S. mansoni* or *S. haematobium* cercariae. No appreciable levels of protection were obtained when the topical antipenetrant was applied to hamster skin at -7 days before exposure to the same species of schistosome cercariae. The results for efficacy against *S. mansoni* and *S. haematobium* cercariae confirm and extend those observations made previously and indicate that the new formulation is highly active as an antipenetrant against two of the major species of schistosome infective to humans.

The new formulation did not protect mice when applied to their skin at either -1, -3, or -7 days prior to exposure with *S. japonicum* cercariae. These results are at variance with data obtained when the previous formulation was tested in hamsters against *S. japonicum* cercariae. Further studies against *S. japonicum* in hamsters are recommended.

No interactions were observed when the new topical antipenetrant formulation was tested for compatibility with a new improved arthropod repellent being developed by the USAMR&D Command. The results obtained indicate that the topical antipenetrant (TAP) is as effective with or without the presence of the arthropod repellent when used against penetrating schistosome cercariae.

The results obtained when the new topical antipenetrant was tested in *Cebus apella* monkeys at -1, -3, and -7 days before exposure to *S. mansoni* cercariae confirm previous observations of the efficacy of the compound in monkeys as a highly active antipenetrant against skin invasion by schistosome cercariae. The compound is completely protective at -1 and -3 days before exposure, and two out of four monkeys were completely protected at -7 days before exposure to cercariae, thus indicating that the new topical antipenetrant formulation has the potential for providing partial protection even at -7 days before exposure to cercariae.

The highly protective activity found previously in mice for compound BL23702 against *S. mansoni* in mice was not sustained in *Cebus apella* monkeys when administered orally. Further studies for this compound are not recommended.

Studies to determine if the marmoset monkey could be used successfully as a model to study schistosome cercariae penetration dynamics indicate that this animal would not be suitable for this purpose. Based on the results obtained, further experimentation was discontinued.

CONCLUSION

Data resulting from animal studies (hamster and monkey) indicate that the new topical antipenetrant (TAP) formulation provides complete protection when applied to skin at either -1 or -3 days before exposure to either *Schistosoma mansoni* or *S. haematobium* infective larva. The formulation does not provide a consistent pattern of complete protection when it is applied to skin at -7 days before exposure to the infective larva.

The new formulation is ineffective in providing meaningful levels of protection against penetration of skin by *S. japonicum* infective larva when tested in the mouse animal model system.

When the topical antipenetrant is used simultaneously with an anti-arthopod repellent, no interactions occur, thus indicating its safety in this regard.

No further studies are recommended for the nine compounds studied for their oral prophylactic effects due to low levels of efficacy when tested in the mouse animal model system.

Due to its low level of efficacy, no further studies are recommended for the compound tested for oral prophylactic effects in the monkey animal model system.

Figure 1. After the applied solutions had dried, the abdomens of the animals were washed for one hour with running tap water, 25 to 27 cycles.

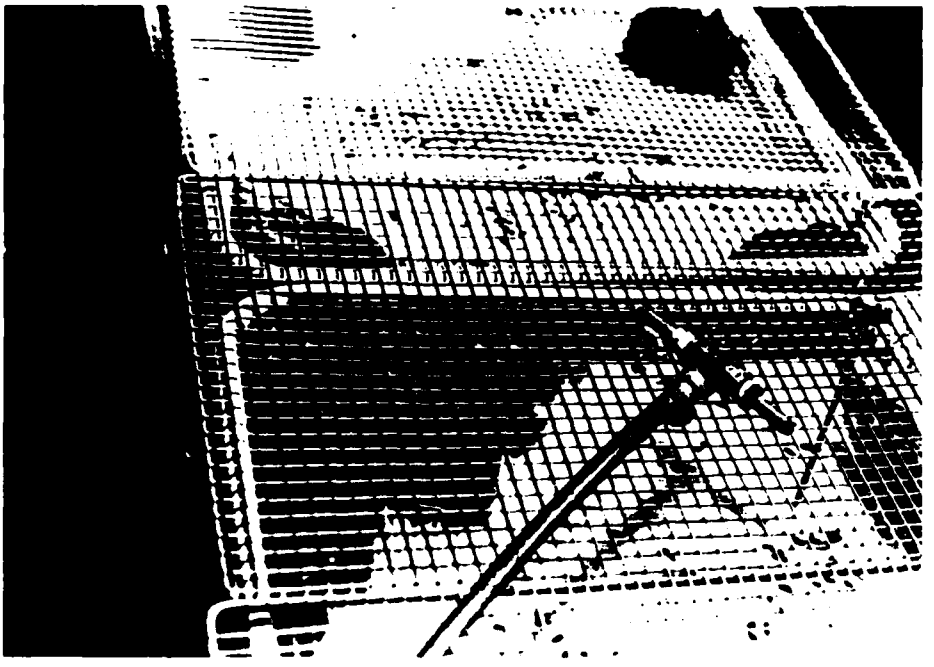

Figure 2. After the applied solutions had dried, the abdomens of the animals were washed for one hour with running tap water, 25 to 27 cycles.

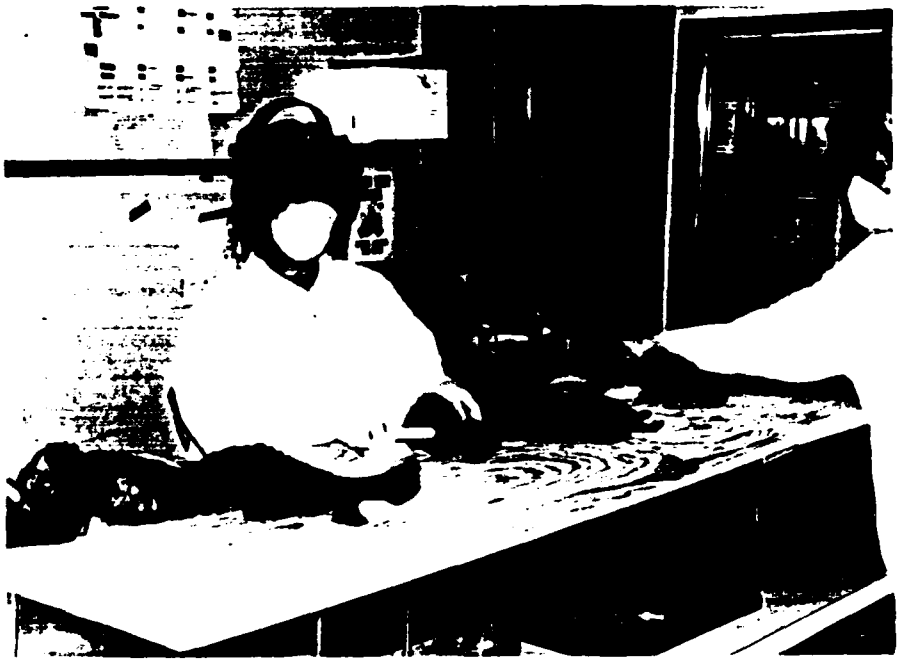

Figure 3. Exposure of *Cebus apella* monkeys to Schistosome infection. Note the hand extended on third monkey on the right. Protective clothing worn during entire procedure.

Figure 4. Exposure of *Cebus apella* monkey to Schistosome infection. Right arm is in container; hand extends through the container.

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FORMS

The data collected will be entered on the Walter Reed Army Institute of Research Anti-Schistosomiasis Drug Screen data forms which have been received along with a description of the codes to be used. The information will be entered onto a data disk and transmitted to WRAIR using a modem and the KERMIT program. If transmission cannot be completed, a copy of the data disk along with a copy of the data forms will be sent to the COTAR at WRAIR:

ATTN: SGRD-WUM-B
Division of Experimental Therapeutics
Walter Reed Army Institute of Research
Washington, D.C. 20307-5011

APPENDIX I*

Techniques for Cultivation and Maintenance of Snail and Schistosome Species and Safety Practices

* Appendix I includes Figures 1 - 5 and Tables 1 - 6

SECTION I

Techniques for Cultivation of *Biomphalaria glabrata* and Maintenance of *Schistosoma mansoni*

I. Units for Snail Maintenance

Two types of units are used.

A. A mobile unit similar to that described by Davis (1971) is used for holding large numbers of breeder and stock (Figure 1). The overall dimensions of this unit are 52 x 175 x 243 cm. This unit is capable of holding 32 glass aquaria (20-liter), 64 plastic trays or 288 petri dishes. There are two types of set-ups for this unit. One is equipped with air lines and fluorescent lights and is used to accommodate glass aquaria for stock snail cultures. Its air manifold system is connected to an oil extractor and air pressure gauge for removing harmful oil droplets from the air and measuring the air pressure, respectively. There are 2 of these units available in this laboratory, which is more than sufficient to meet any *Biomphalaria* culture requirements. The second type of set-up is one in which only fluorescent lights are used for accommodating the type of aquaria (plastic trays and petri dishes) which do not require aeration. This mobile unit is also used for cultivating algae needed as food for snails.

B. A mobile unit, constructed of heavy-duty steel (Figure 2) is used for holding pre-patent snails, patent snails and algal cultures. The overall dimensions of this unit are 61 x 122 x 188 cm. It is capable of holding 50 plastic trays or 180 petri dishes. This unit is set up in two

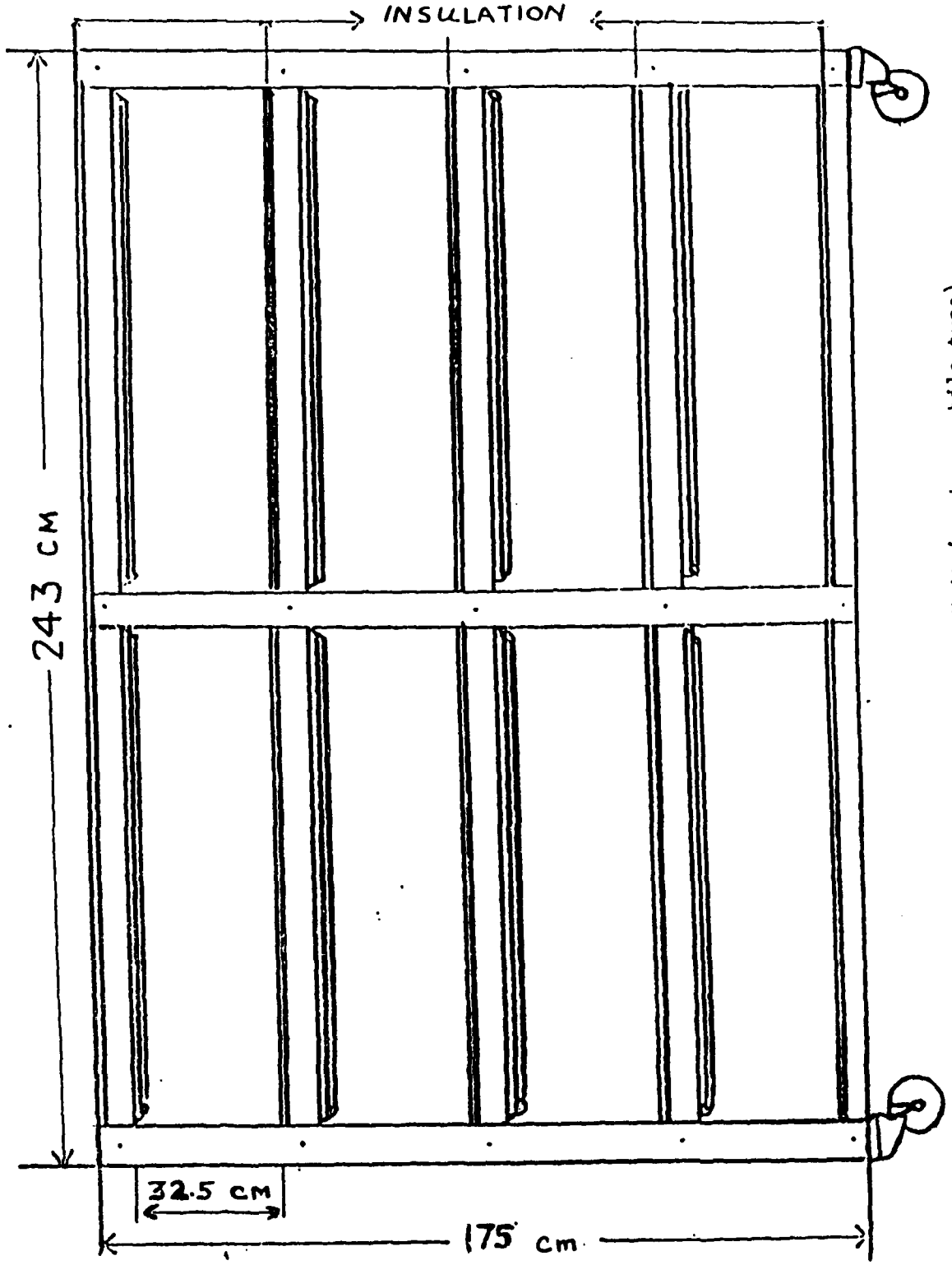

Figure 1 Snail Maintenance Unit (wooden, mobile type)

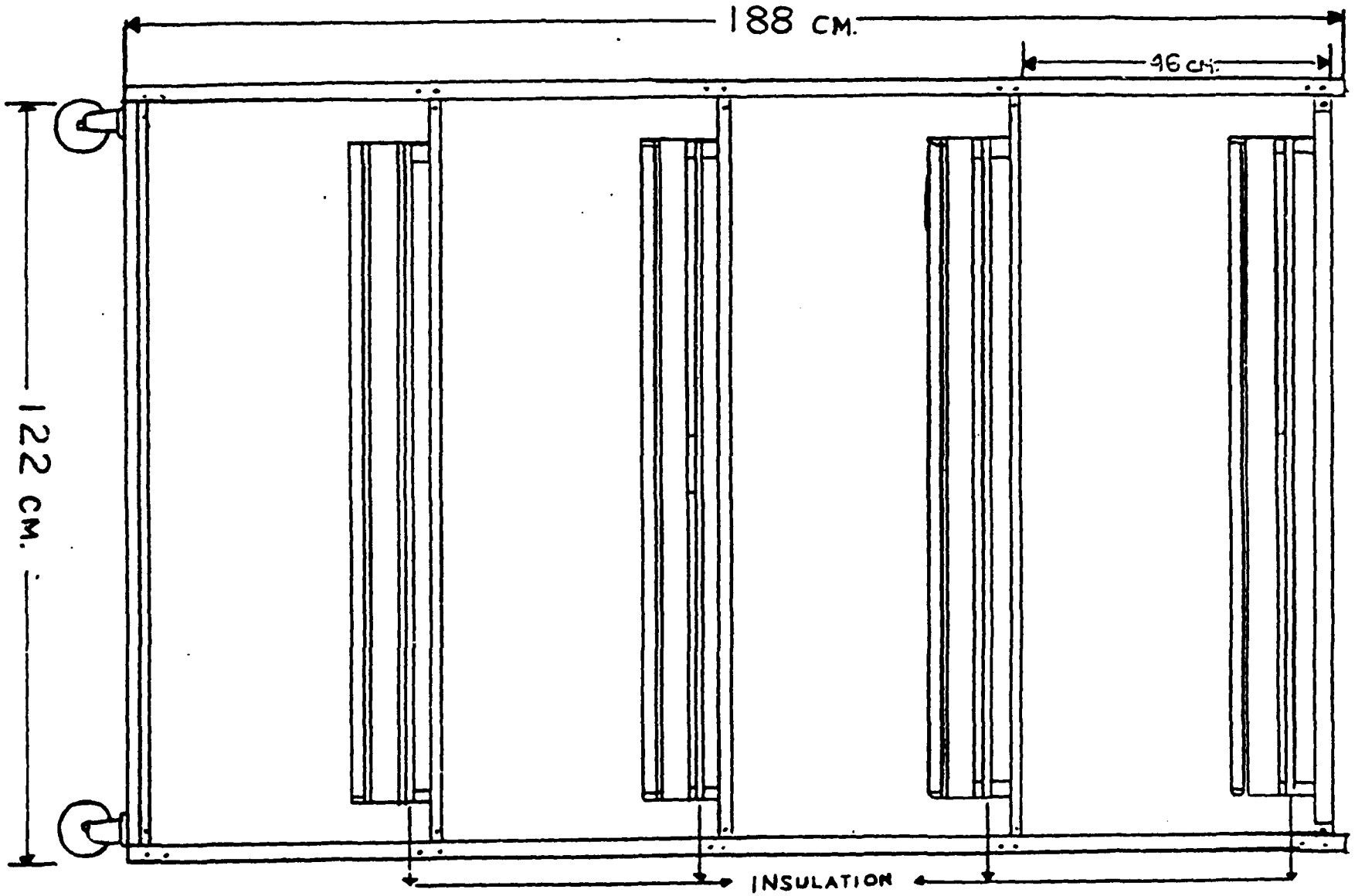

Figure 2 Snail Maintenance Unit (steel, mobile type)

types, neither of which is equipped with air lines. The first type of set-up employs fluorescent light. Four of these are available for accommodating plastic trays for pre-patent snails and petri dishes for algal cultures. The second type of set-up does not employ fluorescent lights. Two of these are available for accommodating plastic trays containing patent snails.

Three kinds of aquaria are used: 1) twenty-liter glass aquaria are used for rearing and maintaining stock snails; 2) plastic trays with a holding capacity of 1.5 liters of water are used as aquaria for breeder snails as well as for both pre-patent and patent snails; 3) petri dishes with a holding capacity of 40 ml of water are used as aquaria for newborn snails.

II. Mouse Restraining Chambers and Exposure Boards

The mouse restraining chambers used for exposing mice to cercarial suspensions by tail immersion technique is a modification of the one described by Stirewalt and Bronson (1955). The exposure boards used for holding the restraining chambers during exposure of mice to cercariae was designed by Loyd (Bruce and Radle, 1971) and is capable of accommodating 100 animals. This board has proven to be most effective for exposing large numbers of animals by tail immersion method to *S. mansoni* cercariae.

III. Environmental Paremeters

A. Light. In both types of snail maintenance units

described above, 40-w "cool" white fluorescent tubes are suspended 12 inches and 18 inches respectively above each shelf. The 20-liter glass aquaria used for maintaining stock snails are held under 12-hour light and 12-hour darkness. The plastic trays (when used for breeding snails and for maintaining pre-patent snails) are placed under constant light.

B. Water. Tap water is conditioned by passing it through a "Diamond" filter (Model #131-1575) containing layers of activated charcoal and sand. This water is then aerated for one day prior to use. The pH of the water is initially 7.1. The pH of the water in glass aquaria and plastic trays is monitored weekly.

C. Aeration. The water of all glass aquaria pans is continuously aerated. The air is supplied by a centrally located air compressor and passes through a water-oil extractor into the culture units. The water contained in plastic trays and petri dishes is not aerated.

D. Temperature. Temperature is monitored daily. A central air conditioning unit maintains the temperature in the snail cultivation rooms between 25°C and 27°C.

E. Food. Romaine lettuce is used as a basic food source. In addition, one or two dishfuls of mud, on which blue-green algae (Nostoc muscorum) has been grown (Liang, 1974) is placed in all glass aquaria, and plastic trays to serve as an additional food source. Snails in petri dishes are given only blue-green algae with mud. In order to improve the growth rate of

snails, an agar mud paste, has been formulated. This paste is found to be consumed very well by Biomphalaria, Bulinus and Oncomelania snails. It contains in addition to the mud, soya bean powder, rice powder, oyster shell powder, cerophyl (dehydrated cereal grass leaves), fish food, yeast and sodium chloride (modified from Moore et al. 1953; Madsen and Frandsen, 1980; and Basch, personal communication, 1984).

IV. Breeding

Snails measuring 12-15 mm are removed from glass aquaria and placed in plastic trays (10 per tray) containing 1.5 liters of aerated tap water and pieces of styrofoams (3 x 5 in.). Trays are placed under continuous light and supplied with food. After one week the trays are changed and the egg masses are removed from the trays and styrofoams, placed in petri dishes with aerated tap water and incubated under ceiling light for about one week until hatching occurs. Newborn snails (0.6-0.8 mm in shell diameter) are transferred with a pipette to petri dishes containing blue-green algae, mud and water and kept at a density of 50 snails per dish. Dishes are maintained under continuous light. At the end of one week, snails are transferred to new dishes with a density of 25 snails per dish. In 14 days, young snails 3-5 mm in size are available for use in either initiating new cultures or for eventual exposure to S. mansoni miracidia.

V. Rearing and Maintenance

For initiating new cultures, snails are removed from petri

dishes in groups of 250 and placed in 20-liter glass aquaria with aerated tap water. For snails which are exposed to miracidia, they are maintained in groups of 50 in plastic trays containing 1.5 liters of water. Patent snails are maintained in plastic trays, 25 snails per tray.

VI. Maintenance of *S. mansoni* Life Cycle

A. Collecting miracidia. Eggs and miracidia are collected according to the method of Liang and Kitikoon (1980). Liver and/or feces-free intestines, excised from mice 8-12 weeks after exposure to *S. mansoni* are used as the sources of eggs. Tissues are cut into pieces and placed into a 200-ml stainless steel Eberbach container with 20 ml of 0.85% saline. The container is placed on a single speed Waring blender (No. 700) connected to a variable autotransformer and tissues are homogenized for 5-10 sec. at very low speed (30 volts). The suspension is poured into a tiered column of sieves arranged in descending order of mesh openings (420 μ, 177 μ, 105 μ, and 45 μ). The eggs are washed through to the bottom sieve with 100 ml of 0.85% saline. Large pieces of tissues, which had been trapped in the top sieves, are re-homogenized in 20 ml of 0.85% saline. The procedure is repeated three times at low (50 volts), intermediate (70 volts) and finally high speed (100 volts). A volume of 100 ml of aerated tap water is poured into the sieve column to rinse eggs free from saline. The eggs are washed from the bottom sieve into a petri dish (2 x 10 cm) with 40 ml of aerated tap water and concentrated to the center of the dish by gentle rotation. The eggs are then pipetted into a small petri dish (1.5 x 6 cm) and

the dish is placed under ceiling illumination for hatching miracidia.

The entire procedure takes approximately 15 min. and miracidia usually appear within 5 min. To insure the high percentage of infectivity, the snails are exposed to miracidia which are less than one hour old.

B. Exposure of Snails. *B. glabrata* snails (3-5 mm in size) are recovered from petri dish cultures and exposed to miracidia with a dose of 5-10 miracidia per snail, either individually or en masse. Individual snail exposures are carried out using 15 x 17 mm glass vials. En masse exposures are carried out using 1.5 x 6 cm-size petri dishes. Each dish contains 5 ml of aerated tap water, 1 cm^2 of blue-green algae and 50 snails. Unless otherwise stated, en masse exposures are used routinely. The temperature for exposure is 25°C - 27°C and the snails are left for 3 - 5 hours in containers under ceiling illumination. After exposure, snails are placed into plastic trays containing 1.5 liters of aerated tap water supplied with lettuce, blue-green algae and mud, and agar mud paste at a density of 50 snails per tray. Snails are maintained under continuous light and they are changed weekly until screening for patency. A total of 1200 snails are exposed monthly. Practically no mortality is to be encountered before patency. From these 1000 to 1100 are to become positive, giving an infection rate of 85% to 90%.

C. Screening Snails for Infection. In order to improve the efficiency in identifying patency of snails, a simple detection method has been devised (Liang and Bruce, in

preparation). Snails exposed 3-4 weeks previously are placed in a petri dish with enough aerated tap water to barely cover their bodies. Under a dissecting microscope, snails are individually examined for the presence of daughter sporocysts. In positive snails the sporocysts, which appear as a whitish mottling in the hepatopancreas, are readily visible through the shells. This method permits selection of 93%-100% of the total snails which ultimately will become positive or negative and is carried out at the rate of 150-200 snails per hour. Subsequently, the positive snails are placed in plastic trays without illumination, and the negative snails are destroyed. Data which support the efficiency and fidelity of this screening method are shown in Table 1. When an early determination of infection is required, it is made by examining the presence of mother sporocysts in the foot muscles and tentacles of the snails at 14 days after exposure to miracidia.

D. Collecting cercariae. Approximately 1200 positive snails are maintained routinely in this laboratory. From these, two types of cercarial collections are made. Type 1 cercarial collection is performed once every two weeks to infect laboratory mice with 150-200 cercariae per mouse for the routine maintenance of S. mansoni cycle. Approximately 100 snails are set out for this purpose. Type 2 cercarial collection is carried out for the purpose of exposing animals for experimental purposes. The number of infected snails to be used will depend on the number of infected mammals to be exposed. Data collected previously on shedding of cercariae from snails provided us with information in predicting the number of snails required for collecting desired numbers of cercariae. Such a prediction model is shown in Table

2. The volume of water required to collect cercariae depends on the dose desired for exposure of mammals. An improved procedure for collection of cercariae has been devised (Table 3). When cercariae are needed, patent snails are removed from the plastic trays and placed in a beaker without water, but with moisture maintained. The beaker is then allowed to stand for 15-20 min. under ceiling illumination at room temperature (25°C-27°C). After this period of time, a small volume of water is added to the beaker and the snails are gently rinsed to remove feces and other debris. This water is then discarded and a second volume of water (100 ml per 100 snails) is added. The beaker is placed uncovered under a 15-w white fluorescent light (18 inches above beaker) for a period of 10-20 min. After this period, the water containing cercariae is decanted into another beaker allowing the snail feces, mucus and other debris to remain in the original vessel. The density of cercariae is then adjusted as follows. The entire procedure takes less than one hour. This procedure dramatically reduces the mortality among snails due to prolonged shedding time.

E. Estimating Cercarial Densities. While mixing the cercarial suspension continuously with a magnetic stirring bar, ten 0.1 ml aliquots are removed from the center of the suspension by a Cornwall continuous pipetting syringe and placed into a 10-cell Boerner counting slide. Before counting under a dissecting microscope, one drop of Lugol's iodine solution is added to each cell. The counts from 10 cells are averaged and the cercarial density is determined. The suspension is subsequently diluted to the desired number of cercariae

per 0.1 ml of suspension before exposure of mammals. For requests which require extremely large number of cercariae per dose, the suspension is passed through a hand-made nylon sieve with 20μ opening to concentrate the cercariae.

F. Exposure of Mammals. Outbred albino mice are used. Three methods of exposures of mammals are available. They are: 1) tail immersion exposure; 2) abdominal skin exposure; and 3) intraperiotoneal injection of cercariae. To insure material for life cycle maintenance, 10 mice will be exposed bi-weekly.

VII. Source and Strain of Snail and Parasite

A S. mansoni parasite and albino Biomphalaria glabrata snail from Puerto Rico and from Brazil (one susceptible to drugs and one resistant to oxamniquine are maintained).

Table 1 Efficacy of detecting daughter sporocysts in hepatopancreas 1638 Biomphalaria glabrata snails (Puerto Rican strain) exposed to miracidia of Schistosoma mansoni (Puerto Rican strain)

Group No.	Days post-exposure	No. of positive and negative snails confirmed microscopically	No. of positive and negative snails confirmed by crushing		% accuracy *
			positive	negative	
1	22	positive 367[a]	367[b]	0	100
		negative 22[c]	0	22[d]	100
2	23	positive 245[a]	245[b]	0	100
		negative 42[c]	3	39[d]	93
3	24	positive 232[a]	232[b]	0	100
		negative 54[c]	4	50[d]	93
4	27	positive 321[a]	321[b]	0	100
		negative 66[c]	2	64[d]	97
5	28	positive 269[a]	269[b]	0	100
		negative 20[c]	5	15[d]	75
Total	-	positive 1434[a]	1434[b]	0	100
		negative 204[c]	14	190[d]	93

* % accuarcy of microscopical method compared to crushing method $= \frac{b}{a} \times 100$ or $\frac{d}{c} \times 100$.

Table 2 Number of Snails Required to Collect Desired Number of Cercariae

Number of snails	Cercarial yield (thousands)
30 - 40	100 - 150
60 - 80	200 - 300
90 - 100	300 - 400
120 - 150	400 - 750

Table 3 Weekly cercarial production of 14 Biomphalaria glabrata(Puerto Rican) snails infected with Schistosoma mansoni(Puerto Rican) under 24-hour lighting

Week No.	No. of cercariae produced by individual snails														Toatl	Mean
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
1	3027	5236	11415	6243	5999	7886	6405	4306	5102	6577	5821	566	4065	2307	74955	5354
2	5575	5658	5496	6091	7383	7120	7229	7095	7470	4565	4827	2601	2575	2830	76515	5466
3	4608	4906	4231	8393	5101	4746	3110	5368	6359	3385	3448	4382	2421	3272	64330	4595
4	1390	2923	2476	4071	1028	3427	3746	4717	3304	2304	1849	3426	1387	2519	38567	2755
5	575	3518	3479	1461	859	4227	3317	4582	2429	2177	3815	4676	4523	3246	42884	3064
6	2246	9942	9597	6449	3009	4304	4961	5818	6512	8503	6812	8650	3616	4502	84921	6066
7	2281	5030	6640	2249	5188	4814	3703	2342	2008	4208	4409	5354	4566	4772	57564	4112
8	2511	5321	7447	4475	2121	6988	8104	5090	3209	7362	9954	6673	5159	5180	79594	5686
9	1185	1054	5255	1449	1076	4435	6713	9214	3069	5132	3002	4386	1947	5177	53094	3793
Total	23398	43588	56636	40881	31764	47947	47288	48532	39462	44213	43937	40714	30259	33805	572424	40888
Mean	2600	4844	6293	4543	3530	5328	5255	5393	4385	4913	4882	4524	3363	3757	63603	4544
Snail sex	♂	♂	♂	♀	♂	♂	♀	♀	♂	♂	♀	♀	♀	♀	-	-

Snails were mono-miracidially exposed except Snail No. 4 which had a bisexual infection. The snails were maintained in Nostoc muscorum algal dishes, one snail per dish. Dishes were changed once a week when cercarial shedding was made. Covering of snails under darkness was not made. Snails were dried for 30 min. prior to shedding.

SECTION II

Techniques for Cultivation of *Bulinus truncatus truncatus* (Egyptian Strain) and *Bulinus truncatus rohlfsi* (Ghanian Strain) and the Maintenance of *Schistosoma haematobium* (Egyptian and Ghanian Strains)

I. Unit for Snail Maintenance

Two types of units are used.

A. A mobile unit similar to that designed by Davis (1971) is used for accommodating large numbers of snails (Figure 1). The overall dimensions of this unit are 52 x 175 x 243 cm. It is equipped with air lines and fluorescent lights and is used to accommodate glass aquaria holding stock *Bulinus* snail cultures. This unit is capable of holding 32 glass aquaria (20-liter size), 64 plastic trays or 288 petri dishes.

B. A mobile unit constructed of heavy-duty steel (Figure 2). The overall dimensions of this unit are 61 x 122 x 188 cm. It is equipped either with or without fluorescent lights. The unit equipped with fluorescent lights is used for accommodating the type of aquaria (plastic trays and petri dishes) which do not require aeration. This type of mobile unit is also used for cultivating algae needed as food for snails. The unit without fluorescent lights is used for accommodating patent snails. Each of these units is capable of holding 50 plastic trays or 180 petri dish cultures. There are five of these units available in this

laboratory, which is more than sufficient to meet the requirements for this proposal.

Three kinds of aquaria are used: 1) twenty-liter glass aquaria are used for maintaining stock snail cultures; 2) plastic trays with a holding capacity of 1.5 liters of water are used as breeding aquaria for collecting eggs and for maintaining pre-patent and patent snails; 3) petri dishes with a holding capacity of 40 ml of water are used for incubating and hatching eggs. Newborn snails are also kept in the petri dishes until they reach 3-4 mm in size.

II. Environmental Parameters

A. Light. The 20-liter glass aquaria used for maintaining stock snails are held under 12-hour light and 12-hour darkness in Type 1 units. The fluorescent light is suspended 12 inches above. The plastic trays (when used for breeding and for maintaining pre-patent snails) are placed in continuous artificial light provided by a 40-w "cool" white fluorescent tube suspended 18 inches above each shelf. Likewise, the petri dishes containing newborn snails are placed under similar light. Plastic trays with patent snails are maintained without illumination. Petri dishes containing egg masses are placed under ceiling light.

B. Water. Tap water is conditioned before use by passing it through a "Diamond" filter (Model #131-1575) containing layers of activated charcoal and sand. The water is then aerated for at least one day prior to use.

The pH of the water is approximately 7.1. The pH of the water in the aquaria and plastic trays is monitored weekly.

C. Aeration. Air supplied to glass aquaria is passed from a centrally located air compressor through a water-oil extractor into culture units. The water contained in plastic trays and petri dishes is not aerated.

D. Temperature. Temperature is monitored daily. It is maintained at 25°C-27°C.

E. Food. Romaine lettuce supplemented with blue-green algae (Nostoc muscorum) and mud forms the diet of stock snails in glass aquaria as well as those kept in plastic trays. The agar mud paste recipe described as an additional food source for Biomphalaria snails is also used for these species of snails.

III. Breeding

Adult snails are transferred to plastic trays containing 1.5 liters of aerated tap water and maitained at a density of ten snails per tray. Trays are placed under continuous light and supplied with food as described above. Trays are changed weekly, at which time egg masses are scraped from the wall of the trays as well as from the surface of lettuce.

IV. Hatching of Eggs and Maintenance of Newborn

Egg masses are placed in petri dishes with aerated tap water and incubated under ceiling light for one week until hatching. Approximately 50 newborn (0.6-0.8 mm in shell length) are transferred with a pipette to petri dishes with blue-green algae and mud, and are placed under continuous illumination. At the end of one week, snails are transferred to new dishes, 25 per dish. Within 14 days, young snails will reach 3-4 mm in size which is suitable for exposure to miracidia.

V. Maintenance of S. haematobium Life Cycle

A. Collecting Miracidia. The large intestines are excised from golden hamsters (Cricetus auratus) with S. haematobium infections of at least 120 days duration. The portion of the intestine having masses of egg nodules is severed and rendered free of feces by rinsing with 0.85% saline. The intestine is cut into pieces approximately 1 cm long and placed into a 200-ml stainless steel Eberbach container with 20 ml of 0.85% saline. The container is placed on a single-speed Waring blender (No. 700) connected to a variable autotransformer and the tissues are homogenized for 5-10 sec. at very low speed (30 volts). The suspension is poured into a tiered column of sieves arranged in descending order of mesh openings (420 μ, 177 μ, 105 μ, and 45 μ). The eggs are washed through to the bottom sieve with 100 ml of 0.85% saline. Large peices of tissues which had been trapped in the top sieves are

re-homogenized in 20 ml of 0.85% saline. The procedure is repeated three times at low (50 volts), intermediate (70 volts) and finally high speed (100 volts). A volume of 100 ml of aerated tap water is poured into the sieve column to rinse eggs free from saline. The eggs are washed from the bottom sieve into a petri dish (2 x 10 cm) with 40 ml of aerated tap water and concentrated to the center of the dish by gentle rotation. The eggs are then pipetted into a small dish (1.5 x 6 cm) and the dish is placed under ceiling illumination for hatching miracidia.

The entire procedure takes approximately 15 min. and miracidia usually appear within 10 min. Snails are exposed to miracidia which are less than one hour old.

B. Exposure of Snails. Snails (3-4 mm in size) are recovered from petri dish cultures and are exposed either individually or en masse. For individual exposures, snails are placed in glass vials (15 x 17 mm) containing 0.2-0.4 ml of aerated tap water. Each snail is exposed to 5-10 S. haematobium miracidia. En masse exposure is carried out using a petri dish (1.5 x 6 cm) containg 5 ml of aerated tap water and 1 cm^2 of blue-green algae at a density of 50 snails per dish. An average of 5-10 miracidia per snail is used. Unless otherwise requested by investigators, en masse exposures are used. The exposure temperature is 25°C-27°C and the snails are left for 3-5 hours in the exposure dishes under ceiling illumination.

After exposure, the snails are maintained for 3 to 4 additional days in new petri dishes with blue-green

algae and mud, 25 per dish. This step reduced the mortality of snails to nil. Thereafter, snails are maintained in groups of 50 per plastic tray containing 1.5 liters of aerated tap water and supplied with lettuce, blue-green algae, mud, and agar mud paste. They are placed under continuous light and the trays are changed weekly until they are screened for infection.

C. Screening snails for Infection. The technique used for screening Bulinus snails for the presence of infection is essentially the same as that described for screening Biomphalaria snails. Snails exposed to miracidia 4 weeks previously are placed in a petri dish with aerated tap water sufficient to just cover snail bodies. Under a dissecting microscope, snails are individually examined for the presence of daughter sporocysts visible through the snail shells. The sporocysts appear as whitish mottling in the hepatopancreas of snails. This method permits selection of 81%-100% of the total snails which ultimately will become positive or negative and is carried out at the rate of 50-75 snails per hour. The positive snails are then placed in plastic trays without illumination. The remaining snails, which show no signs of infection by this method, are re-examined 2 weeks later by the shedding method. Snails are kept in darkness for a period of 16 hours prior to inducing them to shed. The snails are then placed individually in glass vials (20 x 30 mm) without water for a period of 30 min. under ceiling illumination at room temperature ($25^{\circ}C$-$27^{\circ}C$). After this period, 5 ml of aerated tap water is added to each vial and the vials are then placed under 15-w white fluorescent lights (18 inches above vials) for 2 hours. With the aid of a dissecting

microscope, vials are examined for the presence of cercariae. Snails found to be negative are destroyed. Data which support the efficiency of this screening method is shown in Table 4. Early detection of infection is made by examining the presence of mother sporocysts in the foot muscles of the snails. Unlike those found in Biomphalaria snails, the mother sporocysts of S. haematobium (Egyptian strain) are found exclusively in foot muscles. Snails 14 days after exposure are placed on a moist petri dish (2 x 10 cm) and allowed to attach their feet on the surface of the dish. The dish is then inverted and the presence of the mother sporocysts is examined through the glass of the dish under a dissecting microscope by adjusting the microscope light. The sporocysts appear as opaque white specks embedded in the foot muscle.

D. Collecting Cercariae. Approximately 150 positive snails of each snail species infected with each S. haematobium strain are routinely maintained. Two types of cercarial collections are performed from these snail populations. Type 1 cercarial collection is performed monthly in order to infect hamsters with 400 cercariae for the routine maintenance of S. haematobium. About 50-70 snails are set out for this purpose. Type 2 cercarial collection is carried out for infection of animals for experimental purposes.

For collection of S. haematobium cercariae, the method used for Biomphalaria snails is also used, except that the patent snails are held in darkness in plastic trays for a period of 16 hours or more prior to shedding. They are then transferred from the plastic trays into a

Table 4 Efficacy of detecting daughter sporocysts in hepatopancreas of 445 *Bulinus* snails exposed to miracidia of *Schistosoma haematobium*

Species and strain* of snail	No. of positive and negative snails confirmed microscopically	No. of positive and negative snails confirmed by crushing		% accuracy **
		positive	negative	
B.t. truncatus (Egyptian)	positive 13[a]	13[b]	0	100
	negative 42[c]	0	42[d]	100
B. guernei (Gambian)	positive 98[a]	98[b]	0	100
	negative 43[c]	8	35[d]	81
B.t. rohlfsi (Ghanian)	positive 171[a]	169[b]	2	99
	negative 78[c]	4	74[d]	95

* Snails used were 4 weeks post-exposure.

** % accuracy of microscopical method compared to crushing method

$= \frac{b}{a} \times 100$ or $\frac{d}{c} \times 100$.

beaker without water, but with moisture maintained. The beaker is then allowed to stand for 30 min. under ceiling illumination at room temperature (25°C-27°C). After this period of time, a small volume of water is added to the beaker and the snails are gently rinsed to remove feces and other debris. This water is then discarded and a second volume of water is added (50 ml/50 snails) and the beaker is placed under 15-w white fluorescent lights (18 inches above beaker) for a period of 2 hours.

E. Exposure of Mammals. The golden hamster (Cricetus auratus) is used as the definitive host for maintaining S. haematobium in the laboratory and is the mammal provided to investigators for S. haematobium infection. The cercarial suspension obtained as described previously is decanted into a petri dish (2 x 10 cm) and placed under a dissecting microscope. Unlike S. mansoni exposure, S. haematobium cercariae are counted individually. Using an extra-finely drawn-out capillary pipette, individual cercariae are transferred onto the moistened shaven abdomen of a hamster which has been anesthetized with sodium pentobarbital and held on a large watch glass (125 mm in diameter). The hamster is exposed for at least 30 minutes. With this technique, the exact number of cercariae used for exposure is determined. Unless requested otherwise, the dose employed is 400 cercariae per hamster. Hamsters used for life cycle are sacrificed and autopsied approxmately 120 days later. For maintenance of the respective S. haematobium cycles, 6-7 hamsters are exposed monthly.

VI. Source and Strain of Snail and Parasite

The Egyptian strain of *Bulinus truncatus truncatus* snail and *Bulinus guernei* (Gambian) snail and *Schistosoma haematobium* (Egyptian strain) originated from the University of Michigan stock. A more recent isolate of *Bulinus truncatus truncatus* and *S. haematobium* from the field in Egypt (1980 and 1981, respectively) is now being maintained and will gradually replace the old stock (snail and parasite) if warranted for use in this program. The Ghanian and Voltan strains of *Bulinus truncatus rohlfsi* and *Schistosoma haematobium* (Ghanian strain) also originated from the University of Michigan stock.

SECTION III

Techniques for Cultivation of the 4 Subspecies of *Oncomelania hupensis* and the Maintenance of the 4 Geographic Strains of *Schistosoma japonicum*

I. Unit for Snail Maintenance

A mobile unit, constructed of heavy-duty steel (Figure 2) is used to accommodate large numbers of snails and cercariae for requesting investigators. The overall dimensions of the unit are 61 x 122 x 188 cm. Two types of units are used, neither of which is equipped with air lines. The unit with lighting is used for accommodating petri dish cultures containing patent, pre-patent, breeder snails and algal cultures. The unit without lighting is used for incubating snail eggs.

Three kinds of aquaria are used: 1) a petri dish (2 x 10 cm) containing peripherally placed mud, blue-green algae (*Nostoc muscorum*) and water is used for breeding and obtaining eggs (French, 1974 and 1977); 2) a petri dish containing a centrally placed mud mount, blue-green algae (*Nostoc muscorum*) and water is used for rearing young snails and maintaining both pre-patent and patent snails (Liang, 1974); 3) a petri dish containing a small amount of blue-green algae (*Nostoc muscorum*), mud and water is used for newborn snails (Liang, 1975).

II. Environmental Parameters

A. Light. Breeder, pre-patent and patent snails are maintained under a 40-w "cool" white fluorescent light with 12-hour light and 12-hour darkness. Eggs collected from breeding dishes are placed in clean petri dishes with aerated tap water and placed on shelves without direct illumination.

B. Water. Tap water is conditioned before use by passing it through a "Diamond" filter (Model #131-1575) containing layers of activated charcoal and sand. The water is then aerated for at least one day prior to use. The pH of the water is approximately 7.1.

C. Temperature. Temperature is monitored daily and maintained at 25°C-27°C.

D. Food. Blue-green algae (Nostoc muscorum) grown in petri dishes with mud (Liang, 1974) is used exclusively as the food source. Newly formulated agar mud paste is used as an additional diet.

III. Breeding

A modification of the culture method described by French (1974 and 1977) is used for cultivating all subspecies of Oncomelania snails. Adult snails (5 males and 5 females per aquarium) are introduced into petri dish aquaria containing peripherally placed mud. After the surface of the mud is washed several times, a small amount of blue-green algae is added. The aquaria are checked weekly

for eggs which are then removed into a petri dish containing aerated tap water. At the same time, snail feces and soiled water are removed and fresh water and algae (if necessary) are added. A small amount of agar mud paste is also added.

IV. Hatching of Eggs and Maintenance of Newborn

Eggs previously placed in petri dishes with aerated tap water are placed on shelves without direct illumination for incubation and hatching. Eggs usually hatch within 18 days. New born snails are then placed in newly established aquaria containing a small amount of blue-green algae and allowed to grow for 2-3 weeks. The snails are maintained under 12-hour light and 12-hour darkness. After reaching 2-3 mm in shell length, the snails are used for exposure to miracidia or for establishing new rearing cultures by placing snails in petri dishes containing centrally placed mud with blue-green algae. A small amount of the newly formulated agar mud paste, as described for use in Biomphalaria cultivation, is also provided for these snails.

V. Maintenance of S. japonicum Life Cycle

A. Collecting Miracidia. Livers and/or feces-free intestines excised from mice exposed to S. japonicum cercariae (pertains to all 4 geographic strains) 8-12 weeks previously are used as the source of eggs to hatch miracidia. Tissues are cut into pieces and placed into a 200-ml stainless steel Eberbach container with 20 ml

of 0.85% saline. The container is placed on a single-speed Waring blender (No. 700) connected to a variable autotransformer and the tissues are homogenized for 5-10 sec. at very low speed (30 volts). The suspension is poured into a tiered column of sieves arranged in descending order of mesh openings (420 μ, 177 μ, 105 μ, and 45 μ). The eggs are washed through to the bottom sieve with 100 ml of 0.85% saline. Large pieces of tissues which had been trapped in the top sieves are re-homogenized in 20 ml of 0.85% saline. The procedure is repeated three times at low (50 volts), intermediate (70 volts) and finally high speed (100 volts). A volume of 100 ml of aerated tap water is poured into the sieve column to rinse eggs free from saline. The eggs are washed from the bottom sieve into a petri dish (2 x 10 cm) with 40 ml of aerated tap water and concentrated to the center of the dish by gentle rotation. The eggs are then pipetted into a small petri dish (1.5 x 6 cm) and the dish is placed under ceiling illumination for hatching miracidia. The entire procedure takes approximately 15 min. and miracidia usually appear within 15 min. If eggs do not hatch within this time period, the eggs are washed again with aerated tap water to facilitate hatching. Snails are exposed to miracidia which are less than one hour old.

B. Exposure of Snails. Oncomelania snails (pertains to all sub-species) 2-3 mm in shell length are recovered from rearing aquaria and exposed to 5-10 miracidia either individually or en masse. Individual snail exposures are carried out using glass vials (15 x 17 mm). En masse exposures are carried out using a petri dish (1.5 x 6 cm) containing 5 ml of aerated tap water and 1 cm^2

of blue-green algae. The temperature for exposures is 25°C-27°C and the snails are left for 3-5 hours in the exposure containers under ceiling illumination.

C. Screening Snails for Infection. Although it is possible to check Oncomelania snails for evidence of infection by placing them in water to shed, the results are often discouraging and this procedure is not routinely practiced in this laboratory. Instead, snails are checked for evidence of patency by using the same basic techniques as described for Biomphalaria and Bulinus snails. Shells of the snails are cleaned and are examined for the presence of daughter sporocysts visible through the shells. The sporocysts appear as whitish mottling in the hepatopancreas of snails. This method permits selection of 85-90% of the total snails which ultimately will become positive or negative and is carried out at the rate of 30-50 snails per hour. These visually-positive snails are cultured until patency. They are then either supplied to investigators or used for maintenance of life cycle. Those visually-negative snails are cultured separately until patency date. They are then crushed at the time the visually-positive snails are used and examined. Data which supports the efficiency of this screening method is shown in Table 5.

D. Collecting Cercariae. Cercariae are obtained by crushing snails in a small petri dish (1.5 x 6 cm) to which is added a small volume (about 10 ml) of water. Unlike the cercariae of S. mansoni and S. haematobium, most S. japonicum (all strains) cercariae swim to the surface of the water and usually remain there. To collect these cercariae a hair-loop is used. The

Table 5 Efficacy of detecting daughter sporocysts in hepatopancreas of 241 Oncomelania hupensis quadrasi (Leyte strain) snails exposed to miracidia of Schistosoma japonicum (Philippine strain)

Group No.	Days post-exposure	No. of positive and negative snails confirmed microscopically	No. of positive and negative snails confirmed by crushing		% accuracy *
			positive	negative	
1	120	positive 19[a]	19[b]	0	100
		negative 31[c]	1	30[d]	97
2	108	positive 61[a]	53[b]	8	86
		negative 79[c]	12	67[d]	85
3	106	positive 5[a]	5[b]	0	100
		negative 16[c]	2	14[d]	88
4	133	positive 10[a]	8[b]	2	80
		negative 20[c]	8	12[d]	60
TOTAL	-	positive 95[a]	85[b]	10	90
		negative 146[c]	23	123[d]	85

* % accuracy of microscopical method compared to crushing method = $\frac{b}{a} \times 100$ or $\frac{d}{c} \times 100$.

hair-loop is fastened to a very fine minute pin, which in turn is attached to a glass tube. The angle of the hair-loop is adjustable. To collect those cercariae which are submerged under the water surface, an extra finely drawn-out capillary pipette is used. For intraperitoneal injection, cercariae are collected by use of a syringe and needle under a dissecting microscope and the dose of cercariae specified by each investigator is injected into mice.

Two types of collections are made for S. japonicum cercariae (pertains to all strains). Type 1 collections are made for exposure of mice used for maintenance of life cycles of the 4 strains. For this purpose, 10-15 snails are used for collection of cercariae. Type 2 collections are carried out when animals are exposed for experimental purposes. The number of infected snails and cercariae to be used for exposure depends on the experimental conditions.

E. Exposure of Mammals. Outbred albino mice are used for maintaining life cycles as well as for providing infected animals to investigators. Two methods of mammal exposures are available. They are: 1) abdominal skin exposures; and 2) intraperitoneal injection of cercariae. An abdominal skin-exposure method is used for both maintaining the life cycles as well as for providing infected mice to investigators unless requested otherwise by the investigators. Exact numbers of cercariae are transferred to the moistened-shaven abdomen of the mouse, which has been anesthetized with sodium pentobarbital, and held on a small watch glass (100 mm diameter). The mouse is exposed for at least 30

min. For maintenance of each of the 4 strains of S. japonicum cycles, 8 mice are exposed monthly with the dose of 30-35 cercariae per mouse.

VI. Source and Strain of Snail and Parasite

Oncomelania hupensis hupensis (Vogel strain) and S. japonicum (Chinese-Vogel strain) are from the University of Michigan stock. O.h. hupensis (Shanghai strain) and S. japonicum (Chinese-Shanghai strain) were obtained through Dr. Mao from the People's Republic of China. O.h. nosophora (Kofu strain) and S. japonicum (Japanese strain) were supplied from Hamamatsu University direct to the University of Lowell. A more recent isolate, O.h. nosophora (Kofu strain) and S. japonicum (Japanese-YYA strain) have been received from the National Institute of Health, Japan, and will gradually replace the older strains.

O.h. formosana (Pu-yen strain) was obtained through Dr. Cross (NAMRU-2) direct from Taiwan and from this laboratory. S. japonicum (Formosan strain) originated from the University of Michigan stock.

O.h. quadrasi (Leyte strain) and S. japonicum (Philippine strain) originated from Leyte, Philippines through Dr. Sano, Hamamatsu University, Japan.

O.h. chiui (Alilao strain) was from the University of Michigan stock and from this laboratory. O.h. chiui (Linco strain) was from this laboratory.

SECTION IV

Routine Maintenance of Snail Laboratory
(applies for all snail species)

Daily checks are made of water levels and the temperature of glass aquaria, plastic trays and petri dishes. Snails which have climbed above the water level are returned to the water. Dead snails and decaying food matter are removed, and mortality among pre-patent and patent snail populations is recorded.

Aquaria containing cloudy water, protozoa, metazoa, etc. are changed immediately. The aquaria are thoroughly soaked with a 10% bleach solution, then thoroughly washed with hot running tap water followed by a final prolonged rinse with aerated tap water (Table 6).

Table 6 Routine laboratory tasks

Tasks	Daily	Weekly
Feed snails	x	
Check temperature	x	
Check level and condition of water in aquaria	x	
Check aeration and filtration system	x	
Check snail mortality; remove dead snails	x	
Record vital statistics	x	
Check pH of water		x
Collect egg masses		x
Set up breeding cultures		x
Set up rearing cultures		x
Change water of cultures holding pre-patent and patent snails		x

SECTION V

Maintenance of Records (applies for all species)

Three types of data recording forms are used to provide continuous monitoring of the production of stock snails and for maintenance of infected snails and mammals. They are designated as: Form 1 (rearing snails); Form 2 (snail infection); and Form 3 (mammal infection). Form 1 is used for recording data pertinent to snail rearing such as date of set-up, numbers of breeders, survivals, eggs laid, and the number and date of young snails produced as well as other pertinent data (Figure 3). Form 2 is used to record conditions of exposure of snails to miracidia, sources of miracidia, survival of snails and number of snails determined to be positive by shedding and crushing (Figure 4). Form 3 is used to record exposure of mammals to cercariae, source of cercariae, survival of mammals, and status of infection at the time the mammals are sacrificed (Figure 5).

With these forms one may monitor the infection parameters of schistosome species from the snail hosts to the experimental mammals. These records are bound periodically and maintained in a separate record room at this facility.

Species: No.

Source:

No. Snail: (♂: ♀:)

Age: Size:

Date Set-up:

Date	No. Alive	No. of				Remarks
		Mass	Egg	E/M	Young	

Figure 3 Form for Rearing Snails

Tray No.:
Date Inf.:
No. Exp.:
Method: en masse_____ ind._____
No. Mir.:
Definitive Host:
Date Inf.
Dose:

Parasite Strain:
Snail:
Age:
Size:
Exposure Time:

Date Examined	No. alive	% surv	Shed		% pos.	Crush		Total % pos.	Remarks
			+	-		+	-		

Date Remarks

Figure 4 Form for Snail Infection

MAMMAL INFECTION

Cage No. _____ Mammal Strain _____

Age _____ Weight _____

Date Exposed _____

Number Exposed _____

Exposure Method _____

Time Start Shed _____

Time Last Mammal Exp. _____

Age of Cercariae _____

Number of Cerc. Counts _____

Aver. Number Cerc. _____

Parasitic Strain _____

Snail Species _____

Date Snail Exp. _____

Number of Snails _____

Number of times previously shed _____

Date	Dead/ Sacrfd	Per-fused	Worms Recovered					Hatch Method	Hatch Time + Results	Snails Exposed		Comments
			Total	Males	Females	Pairs	Imm.			No.	Species	

Figure 5 Form for Mammal Infection

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